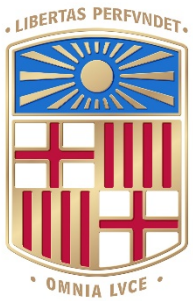

UNIVERSITAT DE BARCELONA

Final Degree Project

Biomedical Engineering Degree

Implementing an optical tweezers setup as a microrheology technique to study the mechanical properties of the cytoplasm

Barcelona, 14th June 2021

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Abstract

Cells in our body are constantly sensing and generating forces through the cytoskeleton. The cytoskeleton is a network of protein filaments that mechanically connects the cells to their substrate and to their nucleus, thus allowing the transmission of mechanical stimuli from the membrane of the cell to the nucleus. More importantly, mechanical forces acting on the nucleus can trigger changes in gene expression and regulate cell behaviour. A recent finding indicates that fibrillar adhesions, a type of cell-substrate adhesions located under the nucleus, alter the transmission of mechanical stimuli from the membrane of the cell to the nucleus, making the nucleus less susceptible to mechanical perturbations. Here, we hypothesise that they do it by tuning the mechanical properties of the cytoplasm. In order to test this hypothesis, we first develop a protocol to acquire microrheological measurements of the cytoplasm with an optical tweezers setup by following three steps: (1) defining a protocol to allow cells to internalise optical tweezers beads in their cytoplasm; (2) establishing a method to acquire microrheological measurements of the cytoplasm with an optical tweezers setup and, furthermore, proving the correct functioning of the system by adding a control that tunes its mechanical properties; (3) implementing a MATLAB program to automatically display and statistically test the data obtained. Once the system is optimised, we measure the mechanical properties of the cytoplasm with and without fibrillar adhesions and we find that cells with fibrillar adhesions exhibit a softer cytoplasm, suggesting a mechanism by which fibrillar adhesions can shield the nucleus from external mechanical perturbations.

Keywords: Mechanobiology, Mechanotransduction, Cell mechanics, Cytoplasm, Nucleus, Fibrillar adhesions, Optical tweezers, Microrheology.

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my research director Amy Beedle for her explanations on every single concept, for her expert guidance during all the steps of this work and for being so generous with her time.

Secondly, I am very grateful to Pere Roca-Cusachs for his advice and for hosting me in his laboratory, the Cellular and Molecular Mechanobiology Group. I would also like to show my sincere thanks to Arnau Farré and his team for being so willing to share their expertise on optical tweezers. In addition, I would like to thank the rest of the people in the Cellular and Molecular Mechanobiology Group and the Integrative Cell and Tissue Dynamics Group, who encouraged me and helped me when needed. Finally, I would like to thank my family and friends for their patience and unconditional support.

Contents

1. INTRODUCTION

1.1. Introduction to mechanobiology

It has been observed that astronauts lose an average of more than 1% of bone mass every month they spend in space (1). The reason why this phenomenon occurs in space and not on earth is because, in space, astronauts experience microgravity or even weightlessness, and it has been demonstrated that the force of gravity is a major determinant of bone remodelling. Without gravity, mechanical demands change, and bone tissue alters its mass and structure in response to it (2).

From this observation one can deduce that, somehow, bone tissue is able to sense mechanical loading, that is, bone tissue is mechanosensitive. Moreover, bone is not the only tissue in which mechano-sensing occurs. Our lungs are stretched every time we breath, our muscles contract every time we move, so does our heart every time it pumps blood, and our vessel walls experience shear stress due to the blood flow (2). All these mechanical stimuli are sensed by each individual cell, the basic unit of life our tissues and organs are made of, and it is in the individual cells that forces regulate essential processes such as proliferation, differentiation, migration and growth, and they are these cellular responses what give rise to the tissue and the organ behaviour (3).

But what happens inside the cells so that a mechanical signal can be converted into a change in gene expression that leads to a certain cell response? How is this regulated and how its alteration can lead to pathological processes? Mechanobiology is a multidisciplinary field of science at the interface between biology, physics and engineering with the aim of providing insights into these fundamental questions, and it is the field in which Cellular and Molecular Mechanobiology Group at the Institute for Bioengineering of Catalonia (IBEC) focuses its work.

1.2. Cellular and Molecular Mechanobiology Group (IBEC)

Cellular and Molecular Mechanobiology Group[1] belongs to the Institute for Bioengineering of Catalonia (IBEC), and it is focused on studying the mechanisms that cells and molecules use to detect and respond to mechanical stimuli. With this aim, they perform experiments combining biophysical techniques such as optical tweezers, magnetic tweezers, Atomic Force Microscopy (AFM) and Traction Force Microscopy (TFM) with cellular biology, optical microscopy and theoretical modelling. The team is very interdisciplinary, it has more than 15 members including the group leader, postdoctoral researchers, PhD students, master students and laboratory technicians coming from engineering, biology and physics backgrounds. I recently joined the group as an undergraduate student to perform the present bachelor's thesis.

Currently, the group has several ongoing projects, but of relevance for this work is the one of Amy Beedle, a postdoctoral researcher in the group. The project aims to unveil the mechanical role of fibrillar adhesions in TIFF fibroblast cells. Fibrillar adhesions are a mature form of focal adhesions that anchor

[1] For more on Cellular and Molecular Mechanobiology Group see: https://ibecbarcelona.eu/cellmolmech

cells to their substrate and are located under the nucleus. One of the recent findings of the project indicates that fibrillar adhesions affect the mechanical coupling between the cell and the nucleus. Specifically, when fibrillar adhesions are present, the nucleus is less susceptible to mechanical perturbations, suggesting that fibrillar adhesions could be protecting the nucleus from being deformed under the action of external mechanical stresses.

At this point, the question that arises is by which mechanism do fibrillar adhesions alter the mechanical coupling between the cell and the nucleus. One of the possible explanations could be that the presence of fibrillar adhesions modifies the mechanical properties of the cytoplasm and this alters the way in which external mechanical perturbations are transmitted to the nucleus. A suitable technique to test this hypothesis is microrheology of the cytoplasm with optical tweezers. However, although the group has already used optical tweezers for other experiments, it is the first time that optical tweezers are used to probe the mechanical properties of intracellular structures. Therefore, addressing the above-mentioned experimental question will require the following steps: (1) define a protocol to allow cells to internalise beads, the mechanical probes of optical tweezers, inside their cytoplasm, (2) optimise a protocol to acquire microrheological measurements of the cytoplasm with an optical tweezers setup, (3) establish a method to display and statistically test the data obtained and (4) measure the mechanical properties of the cytoplasm in the presence and in the absence of fibrillar adhesions and discuss the implications of the results. These are the four steps that the present bachelor's thesis includes.

1.3. Objectives

The main objective of this project is to develop a protocol to study the mechanical properties of the cytoplasm using an optical tweezers setup as a microrheology technique and to test it for two different cell conditions, with and without fibrillar adhesions.

The specific objectives are the following:

1) Develop a protocol to allow cells to internalise beads in their cytoplasm, including a final step to check that the beads are, in fact, in the cytoplasm.
2) Optimise a protocol to acquire microrheological measurements of the cytoplasm with an optical tweezers setup, including a control that can tune the mechanical properties of the cytoplasm and prove the correct functioning of the system.
3) Establish a method to display and statistically test the data obtained with the optical tweezers setup and implement it in a MATLAB program.
4) Measure the mechanical properties of the cytoplasm in the presence and in the absence of fibrillar adhesions and discuss the implications of the results.

1.4. Scope and limitations

Despite being part of a bigger project that aims at unveiling the mechanical role of fibrillar adhesions and that started in 2019 and will continue until 2022, the scope of this bachelor's thesis is temporarily limited as it will be presented in June 2021. Its development has been divided into two time periods. During the first time period, from July 2020 to September 2020, the necessary skills in cellular culture techniques

and optical microscopy techniques were acquired. During the second time period, from February 2021 to June 2021, the present work was performed.

Regarding the activities that this bachelor's thesis includes, it will cover the process of developing a protocol to study the mechanical properties of the cytoplasm using an optical tweezers setup and the process of testing the protocol in two different cell conditions, with and without the presence of fibrillar adhesions. Future experiments that can provide more insights into the experimental question presented are beyond the scope of this project.

1.5. Outline

The present work is structured in four main sections depicted in the following methodology flowchart (Figure 1).

Figure 1. Methodology flowchart.

Theoretical background, described in section 2, provides the theoretical framework of the project. It describes the importance of studying cell mechanics as well as the current state of the situation regarding the study of fibrillar adhesions in the Cellular and Molecular Mechanobiology Group. It also describes the main available tools to study cell mechanics and why microrheology with optical tweezers was chosen as the most suitable technique to study the mechanical properties of the cytoplasm with and without the presence of fibrillar adhesions.

Market analysis, described in section 3, identifies the potential users of the work conducted and recapitulates the evolution of optical tweezers as a microrheology technique in the study of the mechanical properties of the cytoplasm. In addition, it identifies future opportunities and threats that this technique could encounter in the market.

Conceptual engineering, described in section 4, provides a variety of solutions to implement in order to fulfil the objectives of the present work. After discussing the advantages and disadvantages of each solution, a final choice of the ideas selected for further development is presented.

Detail engineering, described in section 5, includes the detail of the implementation of the selected solutions, including a description of the equipment, the materials and the methods used. It also presents the results obtained and discusses their implications.

In addition to these four main sections, the execution schedule, the technical and economic feasibility, the legal aspects and regulations and the conclusions and future perspective are also commented at the end of this report.

2. THEORETICAL BACKGROUND

2.1. Cell mechanics

Cells are constantly sensing and actively generating forces. They do it through the cytoskeleton, a dynamic network of protein filaments that acts as a scaffold in eukaryotic cells. The cytoskeleton is linked to itself, to the plasma membrane and to the nucleus, the largest organelle in the cell and where the DNA is contained. As it can be seen in Figure 2, through its links with the plasma membrane, the cytoskeleton participates in the adhesions that cells use to attach to their substrate. These adhesions are called focal adhesions and act as a mechanical link between the intracellular cytoskeleton and extracellular proteins that are part of cells' substrate. Moreover, as it can also be seen in Figure 2, the connection between the cytoskeleton and the nucleus, mediated by the LINC complex, also acts as a mechanical link, in this case, between the cytoskeleton and the nucleus, and makes the nucleus susceptible to the forces that are transmitted through the cytoskeleton (4).

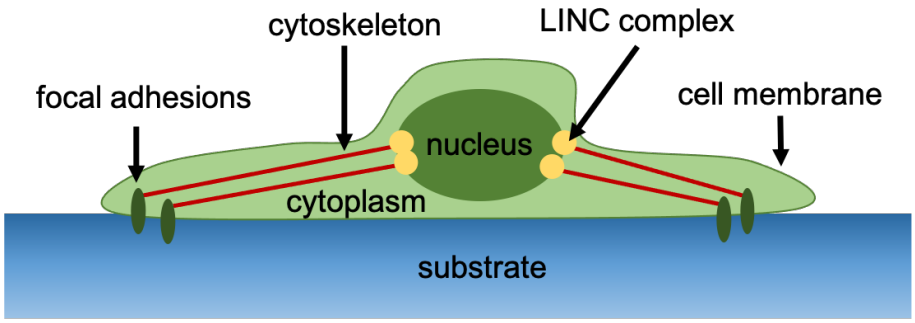

Figure 2. Representation of a cell attached to its substrate and showing the main elements involved in the transmission of mechanical forces from the membrane of the cell to the nucleus.

Therefore, the substrate, the cell and the nucleus behave as a system that is mechanically connected and that is able to transmit mechanical stimuli between its components, interestingly, much faster than any diffusion-based or translocation-based signal propagation (5). Moreover, due to the fact that cells are living organisms and can actively generate forces by contracting its cytoskeleton, the mechanical stimuli that cells transmit and sense are not only determined by external mechanical stresses, but also by the forces that cells actively generate intracellularly.

However, in order to respond to all these mechanical cues, cells need to be able to convert mechanical signals into biochemical signals that can end up changing gene expression and, therefore, cell behaviour. This process is called mechanotransduction and one of the organelles in which it can occur is the nucleus. It has been described that forces acting on the nucleus can alter DNA conformation and, hence, regulate its access to transcription factors and transcriptional machinery that can activate or inactivate the expression of genes (5). In addition, it has also been described that, when forces reach the nucleus, the nucleus deforms and the nuclear pores are stretched, decreasing their mechanical resistance to the transport of particles. This allows the import of molecules that can potentially affect gene transcription, DNA replication and RNA processing (6).

The cellular responses that cells can trigger by means of mechanotransduction are as diverse as proliferation, differentiation, migration and apoptosis, and are essential for organisms' development and homeostasis. Furthermore, the mechanisms involving how forces are generated, transmitted and sensed by cells are finely tuned, and small alterations can lead to pathological processes, including vascular and musculoskeletal disorders, fibrosis and cancer (7–10).

2.2. The mechanical role of fibrillar adhesions

Fibrillar adhesions are a mature form of focal adhesions that cells form at longer timescales, after having pulled some of the focal adhesions towards the centre of the cell through the action of the actin, one of the major components of the cytoskeleton. These mature fibrillar adhesions are associated with the remodelling of the fibronectin, an extracellular protein that is part of cell's substrate, and with the formation of actin fibres beneath the nucleus. Figure 3 shows a schematic of a cell with the addition of fibrillar adhesions and the associated fibronectin remodelling and actin stress fibres (11).

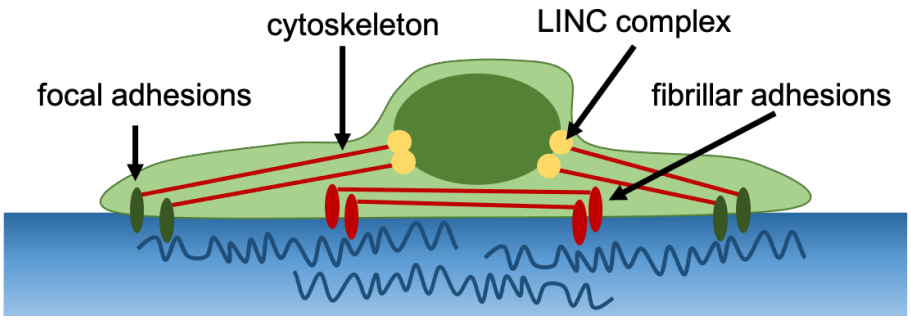

Figure 3. Representation of a cell attached to its substrate and showing fibrillar adhesions and the associated fibronectin remodelling (blue curled lines) and actin stress fibres (red straight lines beneath the nucleus).

More importantly, one of the recent findings in the Cellular and Molecular Mechanobiology Group indicates that the presence of fibrillar adhesions alters the mechanical coupling between the cell and the nucleus. This finding is supported by experiments performed with a cell stretching device. In these experiments, cells are placed on top of an elastic membrane that can be subjected to a uniform and highly controlled strain, while simultaneously imaging the cell response. In typical experiments, there is a high correlation between the cellular strain and the nuclear strain, indicating that the cell membrane and the nucleus are highly mechanically coupled. However, in the case of experiments performed on cells with fibrillar adhesions, the strain on the nucleus is much lower than the strain on the cell, suggesting that fibrillar adhesions stop the deformations applied to the cell membrane from efficiently reaching the nucleus. Figure 3 shows a sketch representing that, by stretching cells the same amount, the nucleus stretches less with the presence of fibrillar adhesions.

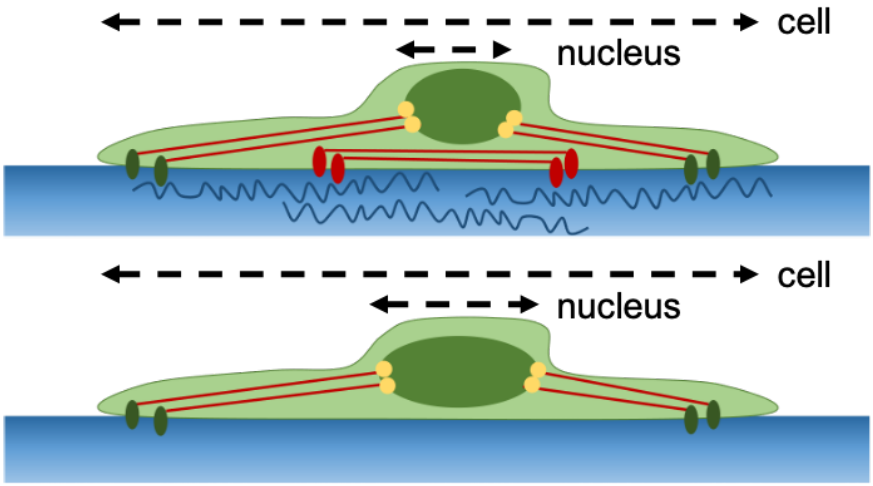

Figure 4. Representation of the stretching experiment with (top) and without (bottom) fibrillar adhesions. The nucleus deforms less with the presence of fibrillar adhesions.

Although the fact that the presence of fibrillar adhesions can protect the nucleus from being deformed can have many implications in cell physiology, the mechanism by which this is regulated remains unknown. One of the possible explanations could be that the presence of fibrillar adhesions modifies the mechanical properties of the cytoplasm and this alters the way in which external mechanical perturbations are transmitted to the nucleus. The reason why fibrillar adhesions could alter the mechanical properties of the cytoplasm is because they are associated with the formation of actin fibres beneath the nucleus, and it has been reported that the cytoskeleton and, especially, the actin cytoskeleton is a major determinant of the mechanical properties of the cytoplasm (12). Therefore, this slight alteration in the actin organisation could tune the mechanical properties of the cytoplasm and alter the transmission of mechanical stimuli from the membrane of the cell to the nucleus. In order to test this hypothesis, the mechanical properties of the cytoplasm with and without fibrillar adhesions, which are also unknown, should be measured. Characterising the mechanical properties of the cytoplasm is particularly challenging since, ideally, it would require a non-invasive technique that can probe the mechanical properties of the interior of living cells. However, in recent years, new biophysical techniques have emerged and have facilitated the understanding of cells' mechanical properties, including the mechanical properties of the cytoplasm (13).

2.3. Tools to study cell mechanics

In order to study cell mechanics, a wide range of biophysical tools that are able to apply or detect forces at the piconewton scale, the scale at which cellular forces operate, have been developed. Very often, they are coupled to optical microscopes, which allow visualising the cells and manipulating them at their scale, the micron scale (14).

Cells are known to behave as viscoelastic materials. However, studies in the past years have revealed that this viscoelastic behaviour depends on the level of mechanical stress applied, the rate of deformation, the loading frequency, the geometry of the probe, the probed location in the cell and the extracellular context, that is, if the cells are adhered to a substrate, floating or forming a monolayer. Therefore, it is

very important to choose the appropriate technique according to the experimental question to be addressed (15).

The following sections present five biophysical tools to study cell mechanics, their advantages and disadvantages and the reasons why microrheology with optical tweezers was considered a suitable technique to study the mechanical properties of the cytoplasm in the presence and in the absence of fibrillar adhesions. A sketch of these techniques is presented in Figure 5.

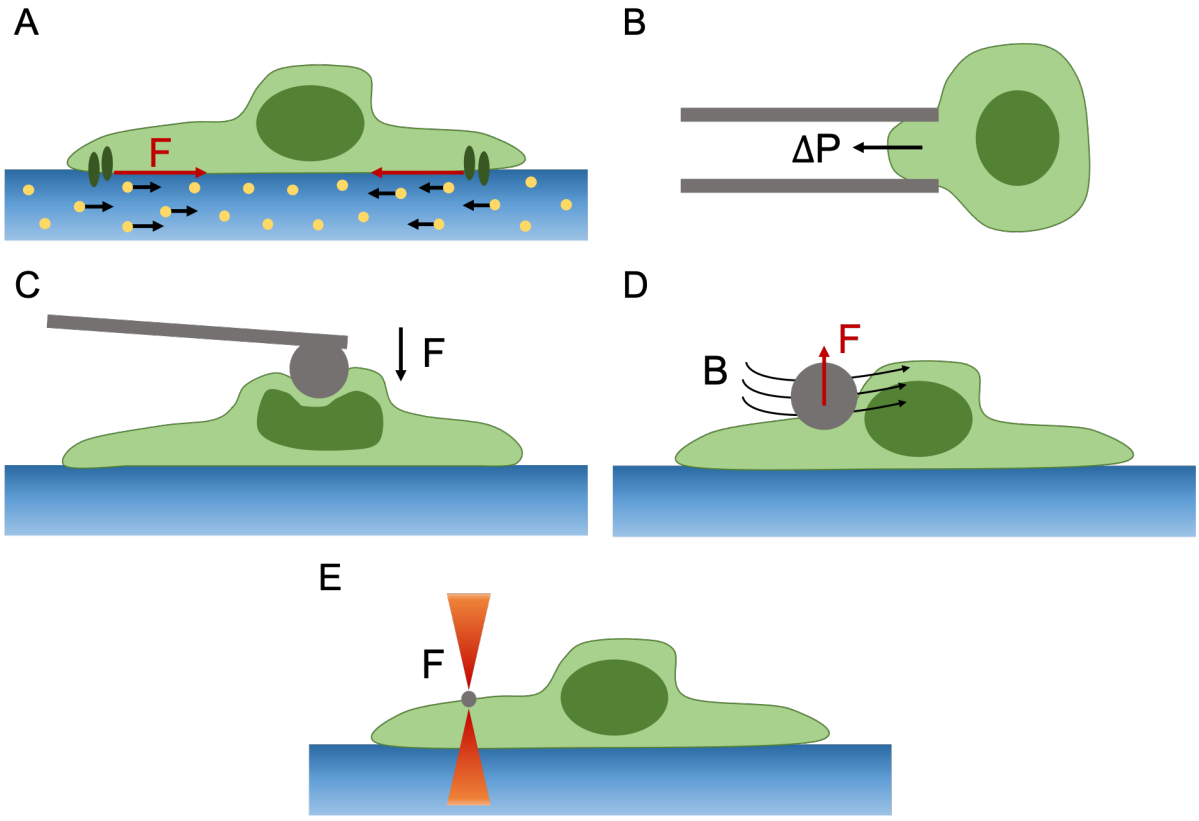

Figure 5. Five biophysical techniques to study cell mechanics. (A) Traction Force Microscopy (TFM), (B) Micropipette aspiration, (C) Atomic Force Microscopy (AFM), (D) Magnetic tweezers and (E) Optical tweezers.

2.3.1. Traction Force Microscopy (TFM)

Cells can adhere to their substrate and exert forces. These forces are called traction forces and can be measured using fluorescent beads embedded in a soft gel, a technique called Traction Force Microscopy (TFM). Fluorescent beads act as fiducial markers and, by tracking their position with and without cells, substrate displacements can be quantified. From the displacements of the gel and knowing its elastic modulus, traction stresses are obtained (14).

TFM has been successfully applied, for instance, to study the impact of the stiffness of the substrate on the forces within focal adhesions and on cell migration (14). However, the force exerted by cells on the substrate serves as an indicator of cell contractility, the mechanical stresses generated by the activity of the actomyosin cytoskeleton, but it does not serve as a direct indicator of the mechanical properties of the cytoplasm.

2.3.2. Micropipette aspiration

Micropipette aspiration is used to study whole cell mechanics by examining how much cellular material is pulled into a glass pipette in response to the application of a negative pressure. From the difference in pressure, the length of the cell inside the pipette and the inner radius of the pipette, the elastic modulus of the cell can be calculated. It is a relatively simple method and is able to provide force resolution down to the piconewton scale. Nevertheless, the spatial resolution is limited to the micron scale since it is based on optical imaging. In addition, the cell can be damaged due to the large deformations it undergoes during the test (14).

Micropipette aspiration has been employed to measure the elastic properties of a great variety of cell types, from chondrocytes to red blood cells and platelets. Furthermore, it has also been used to study single nuclei mechanics by extracting them from cells and testing them with the pipette (14). Nevertheless, the elastic response of the cell measured by this technique depends not only on the mechanical properties of the cytoplasm, but also on the mechanical properties of the cell membrane and the actin cortex, a layer of proteins on the inner face of the cell membrane that is known to be stiffer than the rest of the cytoplasm (13). Moreover, in order to perform this experiment, cells have to be floating, detached from the substrate, and this is not a condition in which fibrillar adhesions can be formed and, hence, it is not a condition in which they can be studied.

2.3.3. Atomic Force Microscopy (AFM)

An Atomic Force Microscope (AFM) consists of a cantilever with a tip at one end that can be used to apply a controlled amount of force on the surface of a cell. The tip can also be functionalised with a ligand, which allows applying forces on specific cellular receptors. The stiffness of the cell can be calculated as the ratio of the applied force over the indentation of the cell. In addition, the probe can be scanned over the whole cell and, therefore, provide a map of the cells' surface elasticity. Furthermore, AFM can also be used as a microrheology technique and apply an oscillatory force over a range of frequencies, which allows calculating the complex shear modulus. It can provide force resolution down to the piconewton scale and position resolution down to the nanometre scale. However, it is limited in probing multiple points of a cell with high temporal resolution and, if scanning forces are very large, cells can be damaged (14,15).

It has been successfully applied, for instance, to study cell mechanics during processes such as mitosis and differentiation (15). However, as in the case of micropipette aspiration, the mechanical response of the cell also depends on the mechanical properties of the cell membrane and the actin cortex, and not only on the mechanical properties of the cytoplasm.

2.3.4. Magnetic tweezers

Both magnetic tweezers and optical tweezers use beads, microspheres, to apply forces through the action of a magnetic field or an infrared laser, respectively, that acts as invisible tweezers. Beads can be functionalised with a ligand to apply forces on specific cellular receptors on the cell surface or can be internalised by cells and probe the mechanical properties of intracellular structures (16,17).

In the case of magnetic tweezers, beads are 1 - 10 microns in diameter and exhibit paramagnetic or ferromagnetic behaviours. Their movement is manipulated by a magnetic field gradient and the force they apply is directly proportional to the intensity of the magnetic field. Forces on the order of tens of nanonewtons can be achieved. Additionally, they can apply an oscillatory force and be used as a microrheology technique. Unlike optical methods, magnetic tweezers induce little heat to the sample and, therefore, less damage. However, displacement, which depends on the viscoelastic properties of the cell, is measured by video-based detection and, therefore, it is limited by the resolution of the optical microscope the system is coupled to, which is on the order of the micron scale. Other disadvantages include the inability to eliminate bead torque or to apply a uniform stress profile to the sample (14,15,17).

Magnetic tweezers have been applied, for instance, in the study of force effect on focal adhesion proteins and cell-cell junctions (14). Furthermore, in the case of studying the mechanical properties of the cytoplasm, they offer more advantages than the above-mentioned techniques. The most important is the fact that magnetic tweezers beads can be internalised by cells and directly probe intracellular structures such as the cytoplasm. Therefore, magnetic tweezers could be an adequate technique to study the mechanical properties of the cytoplasm.

2.3.5. Optical tweezers

In 1970, Arthur Ashkin reported the following: “Micron-sized particles have been accelerated and trapped in stable optical potential wells using only the force of radiation pressure from a continuous laser” (18). Sixteen years later, in 1986, it was finally demonstrated that a tightly focused light beam was capable of holding microscopic beads in three dimensions. Nowadays, this technique is known as optical tweezers, and it has demonstrated to be particularly useful in the study of biological systems (19).

The physical principle behind the optical tweezers relies on the fact that light has momentum, and, in a closed system, momentum is conserved. As Figure 6A illustrates, when a ray of light enters a media of a bigger refractive index such as a bead, its direction of propagation changes following Snell’s law and, therefore, its momentum changes. To compensate for this change in momentum, the bead moves in the opposite direction pushed by a force equal to the change in momentum with respect to time. However, if there is not a single ray but a tightly focused laser beam, that is, a beam whose intensity is very strong in the centre, the optical force will be zero if the bead is placed in the centre of the beam since there will be the same number of rays entering through both sides. Conversely, if the bead leaves the centre, it will be more illuminated through one side than the other and the optical force, which points towards the beam focus, will push it back to the centre (19). This is how a bead can be hold in place, trapped, by light.

Figure 6B shows a bead trapped in three dimensions. The gradient force (F_g) represents the component of the optical force that points in the direction of the gradient of light intensity, whereas the scattering force (F_s) represents the component that points in the direction of propagation of the laser beam. Stable trapping is achieved when the gradient force dominates over the scattering force (19).

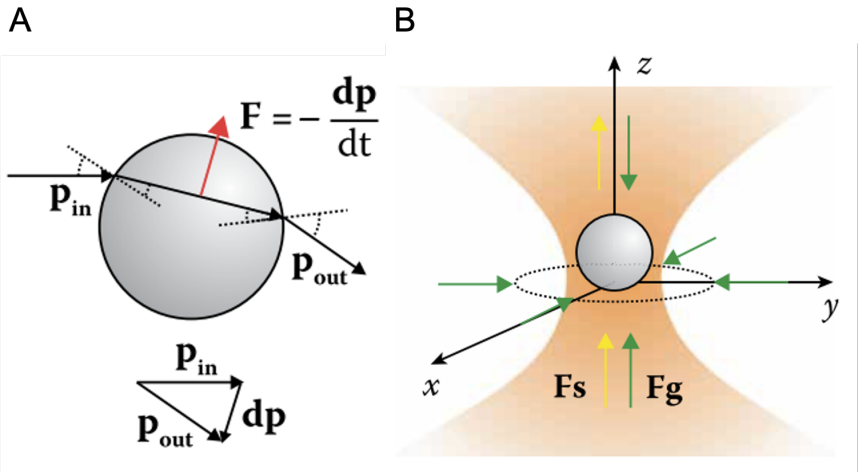

Figure 6. (A) Light rays are refracted two times when they pass through a bead. p_{in} and p_{out} are the incoming and outcoming momentum, respectively, and dp is the difference vector. F is the optical force that the bead experiences. (B) A bead trapped in three dimensions showing the gradient force (F_g) and the scattering force (F_s). Images adapted from (19).

By trapping beads of 0.5 - 5 microns in diameter, optical tweezers are able to produce extremely low forces, from 0.1 to 100 piconewtons, which is ideal for mechanically probing at the cellular and molecular level. They also have high spatial and temporal resolution, less than a nanometre and less than a millisecond, respectively. Moreover, they can apply oscillatory forces and be used as a microrheology technique or create multiple trapping beams and submit the sample to a more complex loading state (14). However, there is a limit on the amount of force that optical tweezers can apply, and the power of the laser cannot be extremely increased since it can induce local heating and damage cells, which would influence their mechanical properties (15).

In addition to being used to probe the mechanical properties of various cell types, optical tweezers have been used to investigate the mechanical properties of proteins and DNA, and to measure the forces generated by molecular motors and RNA polymerase molecules during transcription (19). In the case of studying the mechanical properties of the cytoplasm, they offer the same advantages as magnetic tweezers, including the possibility to internalise beads inside cells and directly probe the mechanical properties of intracellular structures in a non-invasive manner, and the ability to perform microrheological measurements and obtain the complex shear modulus. Moreover, they offer additional advantages such as higher force resolution, higher spatial and temporal resolution and capacity to apply extremely controlled forces. Their only drawback, the inability to produce large forces, will not affect this experiment in particular since the forces needed to disturb the cytoplasm are described to be lower than 100 piconewtons (12). Therefore, optical tweezers are chosen as the most adequate technique to probe the mechanical properties of the cytoplasm.

2.4. Optical tweezers as a microrheology technique

In addition to choosing a suitable biophysical tool to study cell mechanics, it is also important to define the pattern of forces and corresponding deformations that will be applied. As it has already been suggested, a particularly interesting technique is microrheology, which allows obtaining the complex shear modulus of the material being tested at different frequencies. Obtaining the mechanical properties of a material for a range of frequencies instead of for a single frequency is particularly interesting in the case of cells since its viscoelastic behaviour is highly dependent on the loading frequency (15).

Microrheology consists in applying a small amplitude oscillatory shear strain and measuring the resultant shear stress. If the strain amplitude is small, the material remains in equilibrium, and it can be assumed that the stress is linearly proportional to the strain, with the complex shear modulus being the constant of proportionality (Equation 1) (20). The complex shear modulus (G^*), defined as $G = G' + iG''$ where $i = \sqrt{-1}$, allows characterising both the elastic and the viscous response of materials. The real part, G', characterises the elastic behaviour, whereas the imaginary part, G'', characterises the viscous behaviour. Elastic behaviours are associated to solid-like materials, which store energy and exhibit an elastic response, whereas viscous behaviours are associated to fluid-like materials, which dissipate energy through viscous flow. More complex materials such as cells exhibit both solid-like and fluid-like responses and are well characterised by the complex shear modulus.

Equation 1. Microrheology. σ is the stress, γ_0 is the strain amplitude, t is time, ω is frequency.

$$\sigma(t) = \gamma_0 \cdot [G'(\omega) \cdot \sin(\omega t) + G''(\omega) \cdot \cos(\omega t)]$$

However, microrheology assays typically probe homogeneous materials, and the cytoplasm of a cell is far from being homogeneous. In fact, it is a highly heterogeneous and complex environment. The reason why microrheology with optical tweezers can still be used to measure the mechanical properties of the cytoplasm relies on the fact that optical tweezers beads are 0.5 - 5 microns in diameter, and the cytoskeleton mesh size is known to be around 50 nanometres. Therefore, when beads are internalised in the cytoplasm of cells, they are confined by the cytoskeleton mesh and embraced by the rest of the cytoplasmatic molecules, which are much smaller. This allows modelling the cytoplasm as a single phase, homogeneous, viscoelastic material (21). Figure 7A shows a bead in the cytoplasm of a cell and Figure 7B shows a sketch representing a microrheological measurement of the cytoplasm with optical tweezers in which it can be seen that the cytoskeleton mesh and the surrounding molecules are much smaller than the size of the bead.

Furthermore, Figure 7C shows the typical force and bead displacement traces obtained when performing microrheological measurements with optical tweezers at a frequency of 1 Hertz. If the behaviour of the material is totally elastic, the response of the bead is immediate, and the two traces do not show any difference in phase. Conversely, if the material is totally viscous, the phase shift between the force and the displacement is 90º. Therefore, in the case of a viscoelastic material, the phase shift will be in the range 0º to 90º (22).

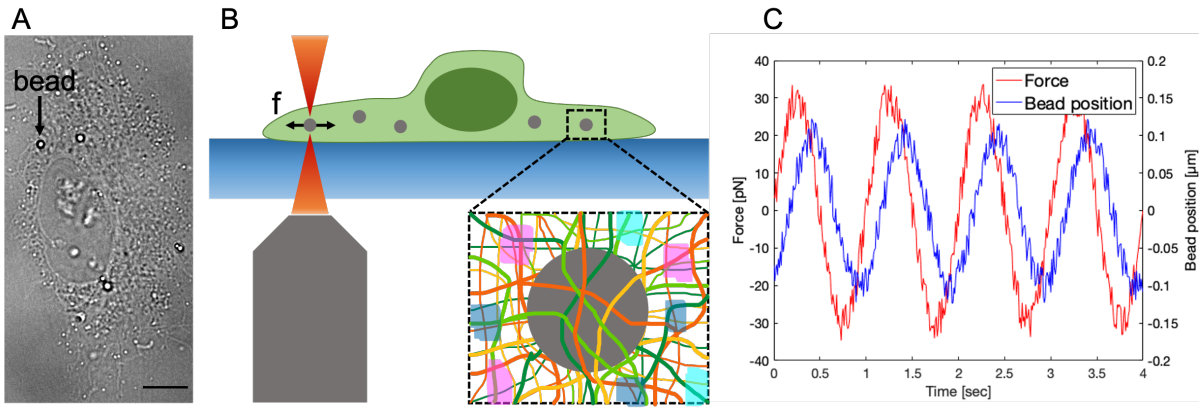

Figure 7. (A) Brightfield image of a 1 µm bead inside a cell. Scale bar = 10 µm. (B) Sketch representing a microrheological measurement of the cytoplasm with optical tweezers. The bead is much larger than the cytoskeleton mesh size. (C) Typical force and bead displacement traces obtained when performing microrheological measurements at 1 Hz with optical tweezers.

It is also worth mentioning that optical tweezers measure the force and the displacement of the bead, and the ratio between them gives a complex spring constant (K^*) with units of Pascals · micron (Equation 2), which indicates the mechanical resistance of the bead moving in a particular media and which it is not the complex shear modulus. In order to obtain the complex shear modulus, which has units of Pascals and is independent of the size of the probe, the complex spring constant must be normalised by a geometric factor derived from the Stokes-Einstein theorem that takes into account the size of the bead, that is, it takes into account that a larger bead will experience more resistance to motion in a viscoelastic media (Equation 3) (23). This is one of the reasons why, although some cellular vesicles present a higher diffractive index than the surrounding media and, therefore, they could be optically trapped, in order to obtain the complex shear modulus, it is necessary to use external beads whose size is well characterised.

Equation 2. Complex spring constant. F is force, x is displacement.

$$K^*(\omega) = \frac{F(\omega)}{x(\omega)}$$

Equation 3. Complex shear modulus. R is the radius of the particle.

$$G^*(\omega) = \frac{K^*(\omega)}{6\pi R}$$

3. MARKET ANALYSIS

This section presents the potential users of the optimised protocol to study the mechanical properties of the cytoplasm with an optical tweezers setup that is developed in this work. In addition, it recapitulates the evolution of optical tweezers as a microrheology technique in the study of the mechanical properties of the cytoplasm. Finally, it identifies future opportunities and threats that this technique could encounter in the market.

3.1. Potential users

The main potential users of the work developed are the members of the Cellular and Molecular Mechanobiology Group. The optimisation of a protocol to study the mechanical properties of the cytoplasm with an optical tweezers setup will allow them to continue studying the mechanical role of fibrillar adhesions as well as other investigations that require accessing the mechanical properties of the interior of living cells.

Furthermore, given the importance of cell mechanics in health and disease, the combined knowledge of cell's mechanical properties and the techniques that can be used to analyse them are always of interest for the healthcare industry. Therefore, this technique could also be used by the healthcare industry, especially for diagnostic applications, as it will be commented in *Future market perspective*.

3.2. Market evolution

The first studies of the mechanical properties of the cytoplasm involving the use of a laser were based on laser-tracking microrheology (LTM), a technique in which a laser is used to track the motion of particles embedded in a particular media with sub-nanometre precision. It is similar to an optical tweezers setup, but, in this case, the laser is only used to track the position of the particles and not to apply and measure forces. Until the end of the 20th century, the spontaneous motion of the tracked particles inside the cytoplasm was assumed to be thermally driven, that is, Brownian motion, and the viscoelastic properties of the cytoplasm were extracted from the measured displacements and using the generalised Stokes-Einstein relationship (24,25).

Nowadays, we understand that cells are systems that operate out of equilibrium and, therefore, the spontaneous motion of particles embedded in the cytoplasm cannot be interpreted as Brownian motion and, hence, cannot be used to infer the mechanical properties of the cytoplasm using the generalised Stokes-Einstein relationship. This is due to the fact that there are many active processes taking place inside the cells that can significantly enhance the spontaneous fluctuation of particles. To overcome this problem, the current applications with optical tweezers actively apply and measure forces on the trapped particles and, from the force and the displacement, the actual mechanical properties of the cytoplasm can be calculated (25).

The current investigations in the field have characterised the mechanical properties of the cytoplasm for various cell types (23,25–27). In all cases, the cytoplasm behaves as a viscoelastic material with a

complex shear modulus that increases as a function of frequency. Moreover, many of these investigations have related the mechanical properties of the cytoplasm to the resistance to motion that molecules and larger complexes experience when they are embedded in it. In 2014, studies from the School of Engineering and Applied Sciences at Harvard University revealed that, in some cancer cell types, the cytoplasmic stiffness is decreased, leading to an abnormally enhanced fluctuation of molecules inside the cells (26). More recently, in 2021, a study from the Institute of Cell Biology at Münster University reported that, during cell division, the cytoplasm of the cell softens, potentially facilitating the correct separation of the chromosomes (23).

Regarding the application for the study of fibrillar adhesions presented in this work, it represents a slightly different approach if compared to the current research in the field since it does not focus on how the mechanical properties of the cytoplasm affect the motion of particles embedded in it, but on how these mechanical properties affect the transmission of mechanical stimuli from the membrane of the cell to the nucleus and, in particular, how this is regulated by fibrillar adhesions, which is still unknown.

3.3. Future market perspective

Optical tweezers as a microrheology technique to study the mechanical properties of the cytoplasm represent a very promising tool in the biological research field that could be used to further characterise and understand both physiological and pathological cell conditions. Furthermore, in a more distant future, they could encounter applications in the healthcare industry, especially in disease diagnosis. There are already commercially available applications of other biophysical tools to study cell mechanics that are used to evaluate the evolution of drug treatments, immune cell activation and cancer prognosis (28). Although optical tweezers are still an expensive and complex technique, they could follow the same path.

4. CONCEPTUAL ENGINEERING

This section describes the proposed solutions that could be implemented in order to optimise a protocol to measure the mechanical properties of the cytoplasm with an optical tweezers setup. After discussing their advantages and disadvantages, the selected solutions that will be actually implemented are presented. According to the objectives, the steps that need to be done are the following: (1) define a method to allow cells to internalise beads in their cytoplasm, including a final step to check that the beads are, in fact, in the cytoplasm; (2) optimise a protocol to acquire microrheological measurements of the cytoplasm with an optical tweezers setup, including a control that can tune the mechanical properties of the cytoplasm; (3) establish a method to display and statistically test the data obtained and implement it in a MATLAB program.

4.1. Study and selection of solutions

4.1.1. Internalisation of beads

The internalisation of beads in the cytoplasm of cells requires defining three important aspects: the size of the beads, the method that will be used to internalise them and, finally, the technique that will be used to check that they are in the cytoplasm.

Size of the beads

When considering what bead size to use, a few aspects need to be taken into account. Firstly, the ability to easily see the beads when they are embedded in the cytoplasm of the cell and, secondly and most importantly, how the bead size influences the stiffness of the optical trap. These concepts are further explained hereunder.

Description of the solutions

The beads suitable for optical tweezers experiments that were available at the Cellular and Molecular Mechanobiology Group were carboxylated polystyrene beads of **1 micron** and **3 microns** in diameter. 1-micron beads are more difficult to distinguish than 3-micron beads when they are embedded in the cytoplasm of cells since there are many intracellular vesicles of a similar size. However, 1-micron beads give rise to a higher optical trap stiffness, which is particularly interesting when measuring the mechanical properties of the cytoplasm at higher frequencies, where the cytoplasm is described to be stiffer (12).

The concept behind the trap stiffness relies on the fact that, for small distances, on the order of the wavelength of the trapping laser, the optical trap behaves as a spring with a certain elastic constant, the trap stiffness. If the bead moves within this distance, the restoring force of the spring places the bead back to the centre of the laser, that is, the bead is trapped. Conversely, if the bead is pushed off this distance, the force rapidly drops to zero and the bead is lost from the trap. Since the higher the trap stiffness, the higher the force that is required to push the bead off the trap, with a higher trap stiffness, larger forces can be applied with the optical trap through the bead without losing it. The trap stiffness depends on many parameters, but there are two of them that can be easily adjusted to match the

experimental needs: the power of the laser and the size of the bead. On the one hand, the higher the power of the laser, the higher the stiffness of the trap. On the other hand, the closer the dimensions of the bead to the wavelength of the trapping laser, the higher the stiffness of the trap (19). Since the laser is infrared (λ = 1.064 microns), 1-micron beads will present a higher trap stiffness and, therefore, they will be more suitable to measure the mechanical properties of the cytoplasm at higher frequencies while maintaining a low laser power that does not damage the cells.

Selection of the solution

The possible solutions regarding the size of the beads were evaluated according to the following criteria:

- Proven to work in similar applications
- Better performance

The possibility to measure the mechanical properties at higher frequencies while maintaining a low laser power was prioritised more than being able to recognise the beads more easily when they are embedded in the cytoplasm. Being able to measure a wide range of frequencies is particularly interesting since the mechanical properties of the cytoplasm heavily depend on the frequency at which they are measured. In addition, although both bead sizes have been described to work in similar applications, 1-micron beads are more widely used, making it easier to compare the results obtained with the literature. Therefore, carboxylated polystyrene beads of 1 micron in diameter are selected.

Internalisation method

The internalisation of beads inside the cells requires a non-invasive method that allows having single beads embedded in the cytoplasm of the cells. There are two methods that have been described to be suitable to internalise optical tweezers beads inside the cells, phagocytosis and microinjection.

Description of the solutions

- **Phagocytosis**: it is the process by which a cell uses its plasma membrane to engulf a particle and it has been described as a suitable method to internalise beads inside the cells. It only requires incubating cells with cell media and beads, and cells engulf beads spontaneously (23,29).
- **Microinjection**: it uses a micropipette to inject substances and particles, and it has been described to work for beads. It usually requires a microscope, a micromanipulator that holds the cells and a micropipette (12,30).

Selection of the solution

The possible solutions regarding the internalisation method were evaluated according to the following criteria:

- Proven to work in similar applications
- Complexity of the process
- Cost

Both phagocytosis and microinjection have been reported to work in similar applications (12,23). However, phagocytosis is simpler and does not require any specific equipment nor any additional costs. In addition, it is a non-invasive method. Therefore, phagocytosis is chosen as the most suitable solution to internalise beads inside the cells.

Characterisation of the localisation

Characterising the location of the beads requires a technique that is able to visualise simultaneously the bead and the cytoplasm in order to determine if the bead is embedded in it. Although with almost any epifluorescent optical microscope it is possible to assess if the beads are inside the cells in the X and Y directions (assuming that the X and Y directions define the planes parallel to the substrate where the cells are seeded), it is more difficult to evaluate if the beads are inside the cells in the Z direction, that is, it could seem that they are inside the cells, but they may be beneath them or on top of them. This is because in an epifluorescent microscope the resolution in the XY plane is much better than in the Z direction. Therefore, two imaging techniques that are able to access the information in the Z direction are proposed as a solution:

Description of the solutions

- **Confocal microscopy**: it is an optical imaging technique that is able to provide high resolution by means of using a pinhole that blocks the out-of-focus light. It allows acquiring images of a very thin plane of the sample for different Z positions, thereby producing a 3D image. The resolution in Z, typically of 0.5 microns, is not as good as in X and Y, typically of 0.2 microns, but it would still allow characterising the location of the beads (31). In order to do so, cells could be fixed and stained for an intracellular structure that colocalises with the cytoplasm, which would allow determining if the beads are within it in 3D. Fixing a sample allows preserving its structure and staining allows fluorescently labelling structures of interest.
- **Transmission electron microscopy**: it is a super high-resolution technique, with a resolution of less than a nanometre, that uses a beam of accelerated electrons as a source of illumination (32). It can be used to study the detailed structure of subcellular organelles and it would allow characterising the exact location of the beads. It would also require fixing the samples and cutting them into very thin cross-sections to allow the electrons to pass through it. It is highly expensive.

Selection of the solution

The possible solutions regarding the characterisation of the localisation were evaluated according to the following criteria:

- Complexity of the process
- Cost

Although, in terms of resolution, transmission electron microscopy is the most suitable technique to determine the exact position of the beads, confocal microscopy provides enough resolution to determine whether the beads are placed in the same Z position than the cytoplasm of the cells. Moreover, the preparation of the samples and the acquisition of the images with transmission electron microscopy would require additional equipment and unnecessary additional costs.

4.1.2. Acquisition of measurements optimisation

The optimisation of the acquisition of measurements with an optical tweezers setup requires the use of a control that is able to tune the mechanical properties of the cytoplasm and prove that the system is functioning correctly. There are several ways in which the mechanical properties of the cytoplasm can be tuned. They are described hereunder as proposed solutions.

Description of the solutions

- **Cytoskeleton drug**: the viscoelastic properties of the cytoplasm are known to be dominated by the cytoskeleton. Therefore, treating cells with a cytoskeleton drug such as blebbistatin, which inhibits the myosin motors and in turn decreases the contractility of the actin cytoskeleton, decreases the mechanical resistance of the cytoplasm. Other cytoskeleton drugs such as cytochalasins, which inhibit the polymerisation of actin, also induce a similar softening effect (12). This can be explained by the fact that the mechanical properties of the cytoplasm are primarily determined by the cytoskeleton and a more disorganised and disrupted cytoskeleton network opposes less mechanical resistance.
- **ATP depletion**: ATP molecules provide energy to drive many processes in living cells, including the contractility of the actin cytoskeleton. Depleting cells from ATP stops all these active processes taking place inside the cells and reduces the mechanical resistance of the cytoplasm even more dramatically than by treating cells with blebbistatin (12,25).
- **Vimentin knockout**: vimentin knockout cells do not express vimentin, one of the proteins that forms the intermediate filaments. Intermediate filaments are another major component of the cytoskeleton, and they also contribute to intracellular mechanics. When intermediate filaments are not present, the intracellular stiffness decreases (27).
- **Osmotic stress/osmotic shock**: applying hyperosmotic stress causes water to quickly leave the cells and it compresses them, leading to an increased cytoplasmic stiffness. In a like manner, applying an osmotic shock, hypoosmotic stress, causes water to quickly enter the cells and reduces its cytoplasmic stiffness (12).

Selection of the solution

The possible solutions regarding the acquisition of measurements optimisation were evaluated according to the following criteria:

- Proven to work in similar applications
- Complexity of the process
- Relation with fibrillar adhesions

All the above-mentioned solutions have been proven to work in similar applications, although the most widely used are cytoskeleton drugs, especially blebbistatin, and ATP depletion. Regarding the complexity of the process, experimenting with vimentin knockout cells would involve the most sophisticated protocols since it requires changing cells' gene expression, whereas the rest of the proposed solutions would involve protocols of similar complexity. However, the on-going project in the research group being conducted by Amy Beedle has already extensively characterised the effect of cytoskeletal targeting drugs on the particular cell line used for this work, TIFF fibroblast cells, which would make easier to put the data

obtained within the framework of already existing data. Moreover, the current hypothesis on how fibrillar adhesions may alter the mechanical coupling between the cell and the nucleus relies on the fact that fibrillar adhesions are associated with the formation of actin fibres beneath the nucleus and this slight change in the actin organisation could alter the mechanical properties of the cytoplasm and the transmission of mechanical stimuli from the membrane of the cell to the nucleus. Therefore, if before the experiment with fibrillar adhesions the system is tested with a control that alters the mechanical properties of the cytoplasm by targeting the actin cytoskeleton, it will be known, specifically, that the system is sensitive to changes in the actin organisation and the whole work would be more robust. Hence, actin targeting cytoskeletal drugs and, specifically, blebbistatin is chosen as the most suitable solution to tune the mechanical properties of the cytoplasm.

4.1.3. Data analysis

Finally, in the current configuration, the experimental data acquired with the optical tweezers setup, the complex shear modulus of the cytoplasm for a range of frequencies, is not provided in a user-friendly way that can be easily manipulated and analysed. Therefore, a pipeline for the data to be properly plotted and statically analysed needs to be developed. One of the main steps involves choosing a suitable statistical test for this type of data. The three proposed solutions are described hereunder.

Description of the solutions

- **Student's t-test of a single frequency**: it is the simplest way to statistically test the data obtained and it would consist in choosing one of the frequencies of the frequency sweep and performing a student's t-test between the two conditions. However, this approach would not take into account all the data nor the fact that there could be statistically significant differences only in some of the frequencies.
- **2-Way ANOVA test**: it can be used to determine whether the differences in the mean of a quantitative variable change according to the levels of two categorical variables, which in this case would be the frequency and the two conditions being tested. Unlike testing a single frequency, it would take into account all the data and, therefore, it would provide a more accurate idea of whether there are statistically significant differences between the two conditions.
- **Fitting the data to a double power law and statistically testing the estimated parameters**: it has been reported that the complex shear modulus of the cytoplasm is well described by a double power law (Equation 4) (34). Therefore, a possible solution would be to fit the two conditions to a double power law and test whether there are statistically significant differences in the estimated parameters ($\mathrm{A}, \alpha, \mathrm{B}, \beta$). In addition, as it has already been described (23), it could be given a physical meaning to the fitted parameters, which would help interpreting the results. This method would also take into account all the data. However, since the statistical test would rely on the fitted parameters, not on the raw data, the fitting should be very robust.

Equation 4. Double power law. G^* is the complex shear modulus, i is $\sqrt{-1}$, f_0 is an arbitrary frequency, f is frequency, A, α, B, β are free parameters.

$$\mathrm{G}^*(\mathrm{f}/\mathrm{f}_0) = \mathrm{A} \cdot (\mathrm{if}/\mathrm{f}_0)^{\alpha} + \mathrm{B} \cdot (\mathrm{if}/\mathrm{f}_0)^{\beta}$$

Selection of the solution

The possible solutions regarding the data analysis were evaluated according to the following criteria:

- Better performance
- Complexity of the process
- Automatable process

The three solutions are automatable processes that can be implemented in a MATLAB program. However, testing a single frequency can be already discarded since it would only give a partial idea of the differences between the two conditions. Regarding the other two solutions, fitting the data and statistically testing the estimated parameters involves a deeper study of the function describing a double power law, the physical meaning that could be given to its parameters and the different ways in which the fitting can be performed to obtain very robust results that can accurately represent the data. All this understanding is beyond the scope of this work and, moreover, it should be done after having understood and tested the raw data. Therefore, a 2-Way ANOVA test is the most suitable solution to test the data obtained and determine if there are statistically significant differences between the different conditions.

4.2. Overall solution

The following modular diagram summarises the selected solutions from the concept generation process (Figure 8). After the implementation of this overall solution, a whole protocol to measure the mechanical properties of the cytoplasm with an optical tweezers setup will be well established and it will be possible to use it to study how the presence of fibrillar adhesions affect the mechanical properties of the cytoplasm. Therefore, this second part of the work will involve following the same protocol but with untreated cells that are able to form fibrillar adhesions and cells treated to prevent the formation of fibrillar adhesions. These protocols are already used in the Cellular and Molecular Mechanobiology Group and, hence, have not gone through this concept generation process.

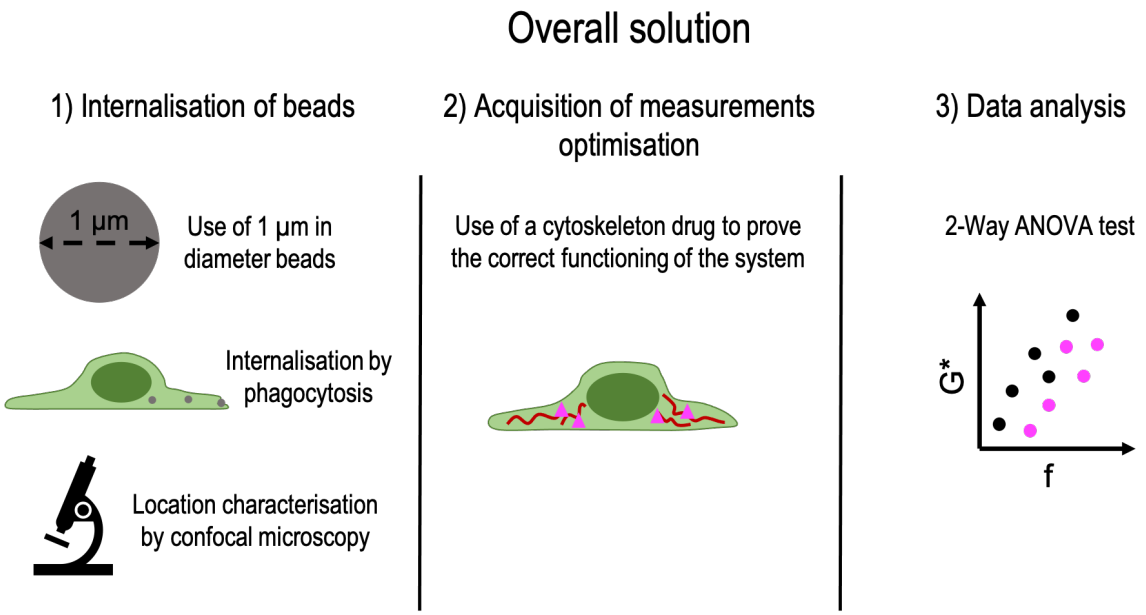

Figure 8. Modular diagram summarising the selected solutions.

4.3. Alternative solutions

In case the selected solutions do not work as expected, alternative solutions could be adopted. If 1-micron beads are very difficult to distinguish when they are embedded in the cytoplasm, fluorescently labelled beads could be used. This would also help the characterisation of their localisation with confocal microscopy. If cells do not spontaneously phagocytise enough beads, beads could be coated with specific ligands that have been described to trigger phagocytosis (35). Finally, if treating cells with blebbistatin does not work as expected, other actin cytoskeleton drugs such as cytochalasin D could be used.

5. DETAIL ENGINEERING

This section explains the detail of the implementation of the above selected solutions as well as the detail of the experiment with fibrillar adhesions. It first describes the equipment and the materials used, then, it focuses on the methods and, finally, it presents the results obtained and it discusses its implications.

5.1. Equipment

The equipment used in this work is divided into two sections. The first one describes the optical tweezers setup and the second one describes additional equipment that was also used.

5.1.1. Optical tweezers setup

In order to perform microrheological measurements of the cytoplasm with optical tweezers, the optical tweezers platform SENSOCELL (Impetux Optics) coupled to an inverted epifluorescence microscope (Eclipse Ti-e; Nikon) enclosed by a heating chamber that kept the samples at 37ºC was used. The optical tweezers platform consists of three modules: the laser source module, the optical trapping module and the force sensor module. The laser source module is located in a rack mount case and contains all the electronics and the infrared laser source (λ = 1064 nm, YLR-5-LP; IPG Photonics). The laser travels through a laser fibre to the optical trapping module. The optical trapping module is assembled onto the microscope body and allows generating and manipulating optical traps through two acousto-optic modulators (DTSX-400-1064; AA Opto-Electronic) that are modulated by a variable frequency driver (Voltage Controlled Oscillator, DRFA10Y2X-D41k-34-50.110; AA Opto-Electronic). After modulation, the beam size is expanded and coupled into the optical path of the microscope from the rear port by a dichroic mirror and focused on the object plane through the objective (Plan Apo VC WI, 60x, NA=1.2; Nikon). To measure the force, the condenser is replaced by the force sensor module (Lunam T-40i; Impetux Optics), which is already calibrated and gives direct access to the force applied by the optical traps on any trapped object.

All the hardware was controlled using LightAce software (Impetux Optics) and, specifically, its Rheology package. In addition, the objective required the use of immersion fluid for water immersion (Zeiss) and the force sensor module required the use of high viscous oil for optical tweezers (Impetux Optics). The manipulation of an infrared laser also required the use of infrared safety googles. Figure 9A presents the microscope with the visible items of the optical tweezers setup as well as the computer with the software. Figure 9B shows the objective used.

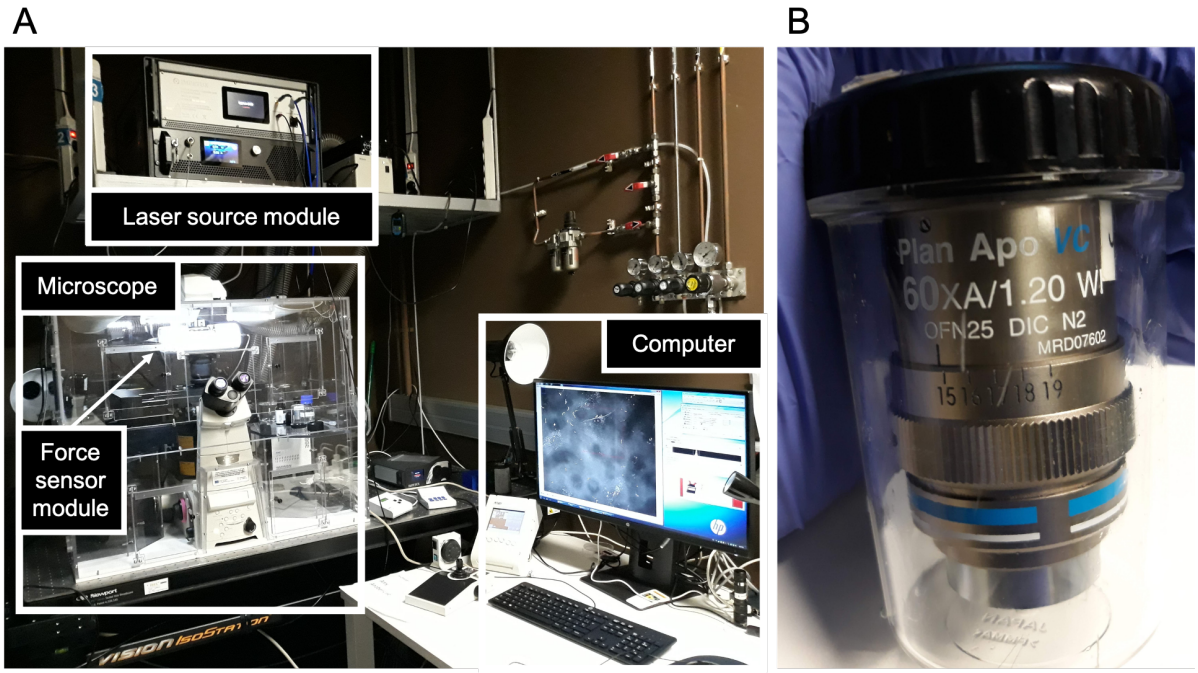

Figure 9. (A) The complete optical tweezers setup including the microscope, the laser source module, the force sensor module and the computer. The optical trapping module is assembled onto the microscope body. (B) The objective: Plan Apo VC WI, 60x, NA=1.2; Nikon.

5.1.2. Other equipment

Other equipment that was required for this work is the following: inverted microscope (Eclipse Ti-e; Nikon) with spinning disk confocal unit (CSU-WD; Yokogawa); cell culture inverted microscope (TS100; Nikon); biological hood (Telstar); cell incubator (Thermofischer Scientific); cell culture centrifuge (Eppendorf Centrifuge 5702); benchtop centrifuge (Eppendorf MiniSpin); ultrasonic cleaner (VWR Ultrasonic Cleaners); fume hood equipped with a nitrogen spray gun (Flores Valles); computer with MATLAB 2021a (MathWorks), Fiji (ImageJ), Mendeley, Microsoft Office and internet access.

5.2. Materials

The materials used in this work are divided into two sections: the materials used for cell culture protocols and the materials used for experimental protocols. Some of the materials used for cell culture protocols were also used in the experimental protocols. In addition, general laboratory materials such as pipettes, syringes, flasks, well-plates, gloves and a lab coat were also used.

5.2.1. Cell culture materials

TIFF fibroblast cells; Dulbecco's modified eagle media DMEM (Thermofischer Scientific) supplemented with fetal bovine serum 10% (FBS) (Thermofischer Scientific), 1% penicillin-streptomycin (Thermofischer Scientific) and 1.5% HEPES 1M (Sigma Aldrich, H0887); CO_2-independent media DMEM (Thermofischer Scientific) supplemented with 10% FBS, 1% penicillin-streptomycin, 1.5% HEPES 1M and 2% L-Glutamine (Thermofischer Scientific); phosphate-buffered saline 1X (PBS) (Sigma Aldrich) 10% in milli-Q water; 0.25% trypsin-EDTA (1X) (Gibco); Neubauer chamber (Blaubrand).

5.2.2. Experimental materials

Carboxylated 1 µm polystyrene beads (Micromod); 24 x 40 mm coverslips with thickness of #1.5 (VWR) (Menzel-Gläser); 12 mm in diameter coverslips (Paul Marienfield); fibronectin (Sigma Aldrich); bovine serum albumin (BSA) 10% in PBS (ThermoFischer Scientific); cytochalasin D (Sigma Aldrich); glutaraldehyde (Sigma Aldrich); paraformaldehyde (PFA) 4% in PBS (ThermoFischer Scientific); Triton X-100 (Sigma Aldrich); fish gelatin (Sigma Aldrich); Alexa Fluor 555 phalloidin (Thermo Fischer Scientific); Hoechst (Thermo Fischer Scientific); mowiol (ThermoFischer Scientific); ethanol 96%; milli-Q water; vacuum grease; 3D printed metal chambers for optical tweezers designed by a previous member of the Cellular and Molecular Mechanobiology Group.

Figure 10 further presents the three main elements that are required to mount a sample for optical tweezers, as it will be described. First, a metal chamber with two inlets on the top view that allow flowing media or drugs once the chamber is closed. Second, two coverslips with #1.5 (VWR) thickness, a suitable thickness for optical tweezers, that are used to close the chamber. Third, vacuum grease that is used to seal the chamber with the coverslips.

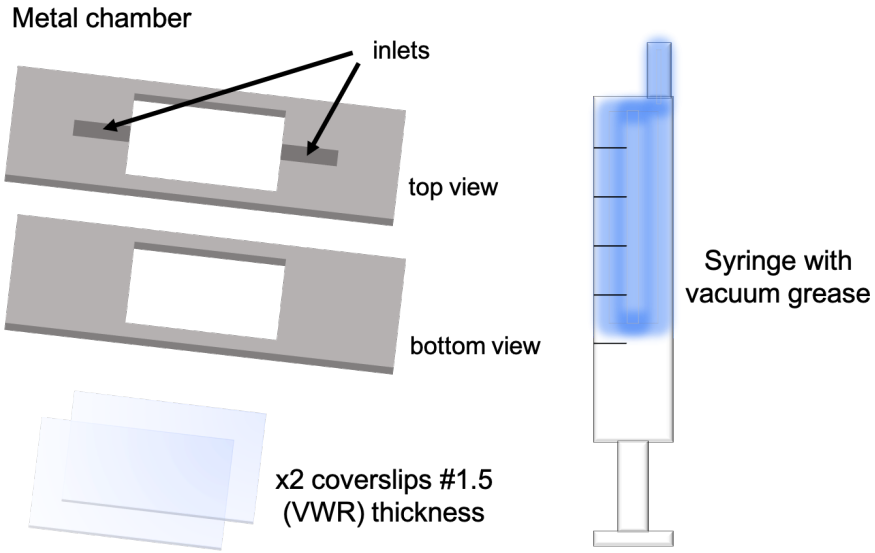

Figure 10. The three main elements required to mount a sample for optical tweezers: a metal chamber, two coverslips #1.5 (VWR) thickness and vacuum grease.

5.3. Methods

The methods used in this work are divided into two sections: the cell culture protocols and the experimental protocols. The cell culture protocols include the steps used to culture cells and to obtain cells for the experiments. The experimental protocols include the steps used to perform all the experiments and are, in turn, divided into four sections: the internalisation of beads; fixing, staining and imaging with confocal microscopy; the acquisition of measurements optimisation with optical tweezers; and the steps regarding the experiment with fibrillar adhesions. It is also worth mentioning that all the steps involving the manipulation of living cells were performed in a biological hood and the stock of cells and the samples with living cells were always maintained at 37ºC and 5% CO_2 in the cell incubator.

5.3.1. Cell culture protocols

TIFF fibroblast cells were cultured with 6 ml of media in a 75 cm^3 culture flask and were passaged twice a week. For the cell passage, the media was aspirated, the flask was rinsed with PBS and cells were incubated for 3 minutes in 1 ml of trypsin to trigger detachment. Once detached, trypsin was neutralised with 1 ml of media and the suspension was centrifuged for 3.5 minutes at 1.3 RCF. Finally, the pellet was resuspended in 1 ml of media and transferred to a new flask. In order to obtain cells for an experiment, after the resuspension of the pellet, the number of cells per ml of media was determined with a Neubauer chamber and the desired number of cells was taken from the resuspension.

5.3.2. Experimental protocols

Internalisation of beads

The steps regarding the internalisation of beads are described below and are also summarised in Figure 11. The number of each step in the text corresponds to the number of each step in Figure 11.

1) 24 x 40 mm coverslips were sealed with vacuum grease on the bottom of the metal chambers. Prior to experiments, the coverslips were sonicated for 5 minutes with ethanol 96% in the ultrasonic cleaner, rinsed thrice with milli-Q water, sonicated with milli-Q water for 5 minutes and dried with a nitrogen gun in the fume hood.
2) Approximately 200 µl of 10 µg/ml of fibronectin in PBS were added on the coverslips for 2 hours at 37ºC. Fibronectin allows cells to attach to the substrate and, moreover, it is required for the formation of fibrillar adhesions. Therefore, functionalising the coverslips with fibronectin allows designing experiments to study the role of fibrillar adhesions.
3) The chambers were sterilised for 10 minutes with ultraviolet light in the biological hood. Then, fibronectin was aspirated, the coverslips were washed thrice with PBS and approximately 200 µl of cell media were added on top.
4) Trypsinised and centrifuged cells were resuspended in 60 µg/ml of beads in media and incubated for 20 minutes. Beads were previously submitted to two short spins with the benchtop centrifuge and resuspended in PBS three times, obtaining a final concentration of 1 mg/ml of beads in PBS.
5) Approximately 15,000 cells were seeded on each chamber and incubated overnight to allow them to phagocytise the beads.
6) The following day, the media was aspirated and replaced by CO_2-independent media since during the measurements with the optical tweezers the samples were not kept at 5% CO_2.
7) Another 24 x 40 mm coverslip was sealed with vacuum grease on top of the metal chambers and CO_2-independent media was flowed through the inlets until the chambers were full.
8) Vacuum grease was added on the edges of the coverslip to prevent the media from covering the top of the coverslip and interfering with the optical path. In addition, a drop of media was added on the inlets to prevent evaporation.

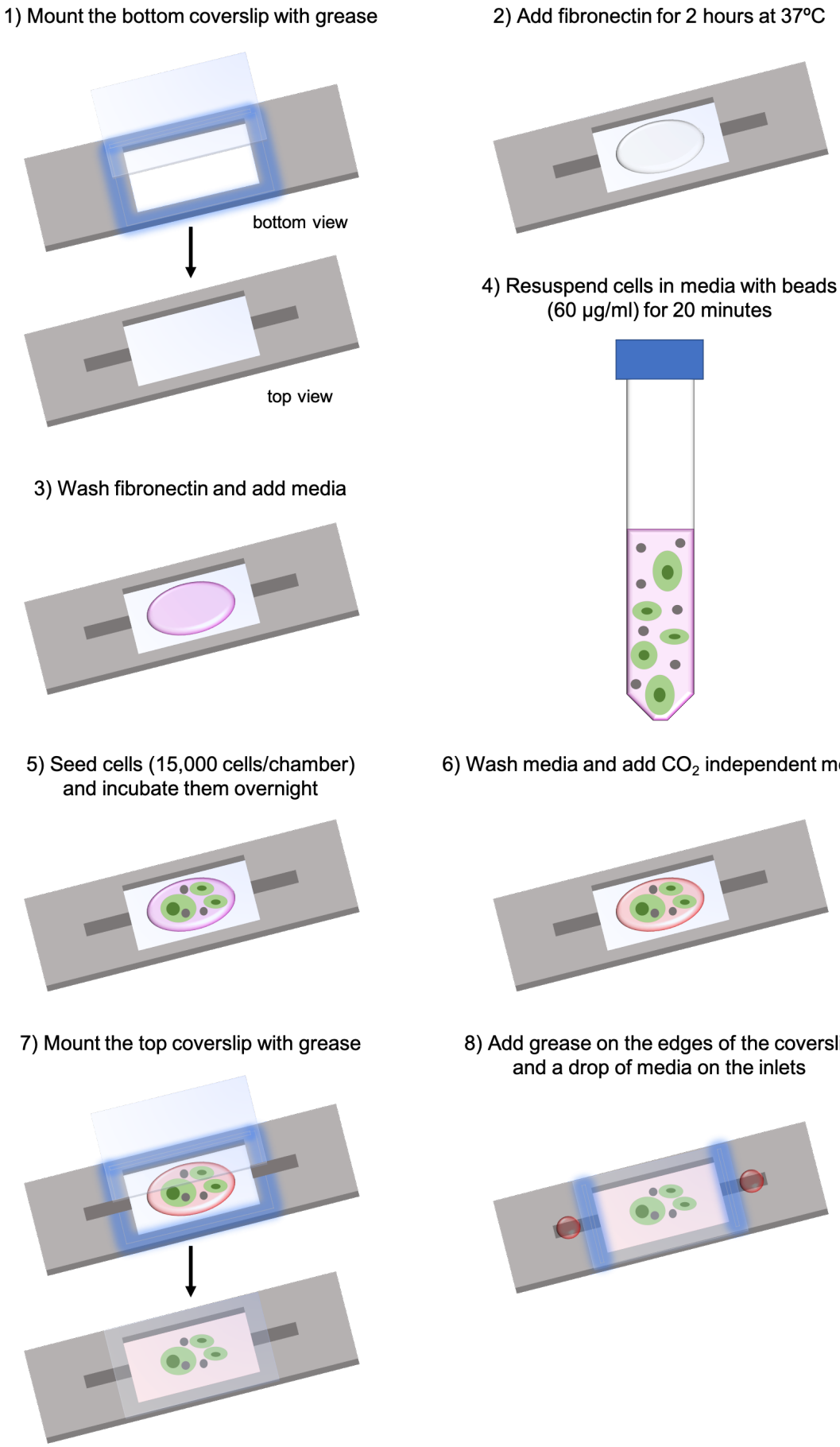

Figure 11. Internalisation of beads protocol.

The steps presented were selected from several trials as the most optimal to allow cells to internalise beads. Other methods that were also tested were resuspending cells in media without beads and adding the beads once cells were seeded and allowed to attach to the substrate, and resuspending cells in media without beads and adding the beads just after cells were seeded, before they were able to attach to the substrate. In addition, the optimal concentration of beads, which was determined to be 60 µg/ml, was also selected from several trials with different concentrations ranging from 10 µg/ml to 1,000 µg/ml.

Fixing, staining and imaging with confocal microscopy

Some of the samples that were prepared for the experiments with optical tweezers were fixed, stained and imaged with a spinning disk confocal microscope in order to determine whether the beads had been internalised by the cells. These samples were taken before adding CO_2-independent media and closing the chamber with the top coverslip, that is, before step 6, according to the protocol described in the previous section. The cellular structures stained were the actin cytoskeleton and the nucleus with fluorescently labelled phalloidin and Hoechst, respectively. Staining the actin cytoskeleton allows identifying the extension of the cytoplasm and, therefore, determining if the beads are within it or not, whereas staining the nucleus allows having a better idea of the global organisation of the cell and the position of the beads with respect to it.

The fixing protocol was performed in the fume hood and included washing the samples once with PBS, adding PFA 4% for 10 minutes to fix them and washing them thrice with PBS. The staining protocol included permeabilising the samples with Triton-X 100 0.1% in PBS for 5 minutes, washing them thrice with PBS, performing two blocking steps with fish gelatin 0.5% in PBS for 15 minutes, incubating them with fluorescently labelled phalloidin 0.1% in PBS for 1 hour; adding the nuclear marker Hoechst 0.1% in PBS for 5 minutes, washing them once with fish gelatin 0.5% in PBS, washing them thrice with fish gelatin 0.5% in PBS for 5 minutes, adding the mounting agent mowiol and placing a 12 mm in diameter coverslip on top. Once mowiol, the mounting agent used to stick the sample to the coverslip, was dried, samples were stored at 4ºC until they were imaged. In addition, after the addition of phalloidin, they were always kept in the dark to avoid photobleaching of the fluorescent molecules, phalloidin and Hoechst.

Finally, samples were imaged with a spinning disk confocal microscope using the same objective as in the optical tweezers setup. For each position in the sample that was imaged, the centre of the cells in the Z direction (assuming that the XY planes are the ones parallel to the plane defined by the coverslip) was determined by finding the plane that presented the maximum intensity for the Hoechst signal, the signal associated to the nucleus. From that central plane, 17 slices (8 above the central plane, 8 below the central plane and 1 in the central plane) separated by 0.5 µm were acquired.

Acquisition of measurements optimisation

The rest of the samples, the ones that were not used to characterise the location of the beads with confocal microscopy, were used to acquire microrheological measurements of the cytoplasm with the optical tweezers setup. Figure 12A shows one of these samples, which would correspond to a sample that completed all the protocol regarding the internalisation of beads. Furthermore, Figure 12B shows how the sample was positioned in the optical tweezers setup. Briefly, the objective was mounted on the microscope and a drop of immersion fluid for water immersion was placed on top. Then, the sample was

secured in the stage holder and the objective was brought into contact with the sample from the bottom. Finally, a drop of high viscous oil for optical tweezers was placed on top of the sample and the force sensor was brought into contact with the sample from top.

Once the sample was mounted on the setup, all the hardware was controlled using LightAce software. Before every experiment, according to the manufacturer procedure, the electronics offset was removed, the power of the infrared laser was uniformised throughout the sample, and its position was calibrated to be the same as the computer mouse. This allowed controlling the position of the optical trap with the computer mouse and placing it at the desired position, in this case, on top of a bead embedded in the cytoplasm of a cell. The total power at the sample was set to 120 mW, which gave rise to a trap stiffness within the range of 190 to 290 pN/µm. Figure 12C shows the user interface of the LightAce software, including the tabs that allow controlling the microscope and the optical tweezers, the tab that allows visualising the sample and the optical trap, and the tab that displays the measurements.

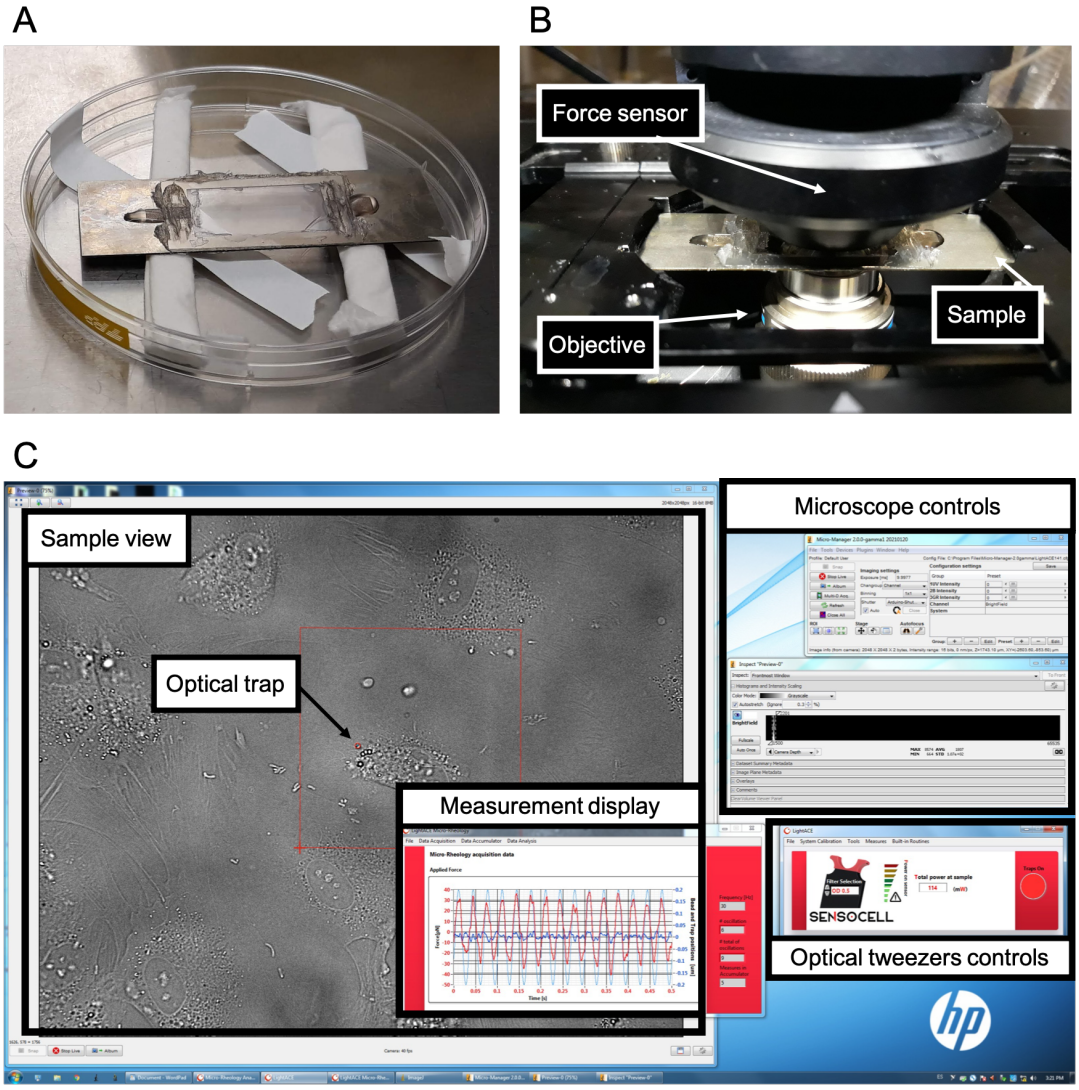

Figure 12. (A) Sample ready to be used with the optical tweezers setup. (B) Sample placed in the optical tweezers setup. Beneath the sample, the objective is touching it through a drop of immersion fluid for water immersion; on top of the sample, the force sensor module is touching it through a drop of high viscous oil for optical tweezers. (C) The user interface of the LightAce software, including the sample view, the optical trap, the measurement display, the microscope controls and the optical tweezers controls.

In addition, before starting the microrheological measurements, which consist in applying a small amplitude oscillatory shear strain at different frequencies and measuring the resultant shear stress, the system required specifying four parameters: the frequencies defining the frequency sweep at which the oscillatory strain is applied, the amplitude of the oscillatory strain, the excitation time and the measuring time. The excitation time corresponds to the time that the oscillatory strain is applied at each frequency, and it is the sum of the measuring time and an extra time that allows the system to adapt to the new frequency before starting to measure. The measuring time corresponds to the time that the system is measuring and, according to the manufacturer, it should be long enough to complete 8 cycles, that is, for a frequency of 1 Hz, at least 8 seconds are required. Table 1 shows the parameters used for this work. The frequency of oscillation ranges from 0.3 to 50 Hz, the amplitude is always 0.2 µm, the measuring time allows completing at least 8 cycles for each frequency and the excitation time includes 1 or 2 extra seconds.

Table 1. Parameters used for the microrheological measurements of the cytoplasm.

Frequency (Hz)	Amplitude (µm)	Excitation time (sec)	Measuring time (sec)
0.3	0.2	29	27
0.5	0.2	18	16
1	0.2	12	10
3	0.2	12	10
5	0.2	12	10
10	0.2	2	1
30	0.2	2	1
50	0.2	2	1

As it has already been mentioned, these measurements were performed on beads embedded within the cytoplasm of cells. However, to avoid measuring the cell cortex' and the nucleus' mechanical properties, which are known to be distinct than the rest of the cell, only beads that were positioned more than 1.5 µm away from the cell boundary and the nucleus were chosen (21). By measuring the force applied by the optical trap on the beads, the position of the beads, and by knowing the size of the beads, the system was able to compute the complex shear modulus at each frequency.

Finally, in order to further prove that the system was functioning correctly, a control that was able to tune the mechanical properties of the cytoplasm was implemented. As it has already been mentioned in *Conceptual engineering*, initially, this control was planned to be the addition of blebbistatin to the sample and the observation of a reduction in the stiffness of the cytoplasm. However, it did not work as expected and the alternative solution was adopted. The alternative solution consisted in using another cytoskeleton drug, cytochalasin D, which both disrupts actin filaments and inhibits actin polymerisation. Therefore, after measuring the mechanical properties of the cytoplasm of untreated cells, approximately 200 µl of cytochalasin D 1 µM in cell media were added thorough the inlets of the metal chamber. After 30 minutes, once cytochalasin D induced its effect, the mechanical properties of the cytoplasm were measured again. The effect expected for cytochalasin D was the same as the one expected for blebbistatin, a reduction in the stiffness of the cytoplasm.

Fibrillar adhesions experiment

At this point, all the steps regarding the optimisation of a protocol to allow cells to internalise beads in their cytoplasm and to acquire microrheological measurements of the cytoplasm with the optical tweezers setup were well established. The next step was to test the same protocol to study the mechanical properties of the cytoplasm with and without the presence of fibrillar adhesions. With this purpose, the protocols previously described were slightly adapted since to prevent the formation of fibrillar adhesions the coverslips needed to be treated prior to the experiments and, hence, cells could not be incubated on the chambers overnight.

Therefore, after being resuspended for 20 minutes in media with beads, cells were seeded on a well-plate instead of on the chambers and incubated overnight. The following day, cells were trypsinised and centrifuged for 2 minutes at 1.2 RCF, less than usual in order to take into account the additional weight of the beads. Finally, cells were seeded on the chambers, which had been previously treated. For the control chambers, approximately 200 µl of 10 µg/ml of fibronectin in PBS were added on the coverslips overnight at 37ºC and replaced by media the following day. The control chambers did not restrict the remodelling of the fibronectin by the cells and allowed them to form fibrillar adhesions. For the condition chambers, the ones that prevented cells from forming fibrillar adhesions, two different treatments were used. After the functionalisation of the coverslips with fibronectin, the first treatment included adding glutaraldehyde 1% in milli-Q water for 15 minutes at room temperature, washing the coverslips with milli-Q water thrice, adding BSA 1% in PBS for 15 minutes at 37ºC, washing them with milli-Q water thrice and adding media. The second treatment included the same steps but, instead of adding glutaraldehyde 1% in milli-Q water, it used PFA 4%. Both glutaraldehyde and PFA treatments are known to prevent the formation of fibrillar adhesions by crosslinking fibronectin and stopping its remodelling.

Regarding the acquisition of measurements with optical tweezers, the same protocol was followed, although the frequency of 0.5 Hz, which gave similar values than the ones given by the frequencies of 0.3 and 1 Hz, was no not acquired.

5.3.3. Data analysis

Finally, the data obtained from the experiments with the optical tweezers was plotted and statistically analysed with a 2-Way ANOVA test to determine whether the differences between the controls and the conditions were statistically significant. All this procedure was implemented in a MATLAB program (see Annexes) that required two inputs: first, the name and the directory of two .txt extension files, one including the measurements of the control and the other one including the measurements of the condition; and second, the number of frequencies of the frequency sweep. In the example presented in Table 1, this number is 8.

The structure of the .txt files obtained from the optical tweezers setup is presented in Figure 13. The files contained five columns, including the frequency, the elastic response function (χ'), the viscous response function (χ''), the elastic shear modulus (G'), and the viscous shear modulus (G''). The viscoelastic response function (χ^*) is equivalent to the inverse of the complex spring constant (K^*) presented in Equation 2 and it was not used for this analysis. The only variable analysed was the complex shear

modulus (G^*). Regarding the number of rows, it was n·m, with n being the number of frequencies of the frequency sweep and m being the number of cells that were analysed.

	Frequency	χ'	χ''	G'	G''
cell 1	f_1				
	...				
	f_n				
...	f_1				
	...				
	f_n				
cell m	f_1				
	...				
	f_n				

Figure 13. Structure of the data obtained from the microrheological measurements of the cytoplasm with the optical tweezers setup. n is the number of frequencies of the frequency sweep and m is the number of cells analysed.

From the information given, the MATLAB program performed the following steps for each type of experiment:

1) The two .txt extension files were read, and, since the complex shear modulus is known to present a log-normal distribution, the mean and the standard error of the log-transformed data of all the independent data sets was calculated. The independent data sets are the elastic and the viscous shear modulus of each frequency and of both the control and the condition. In addition, all the independent data sets were plotted in a loglog scale showing the mean and the standard error.
2) The mean and the standard error of the log-transformed ratio between the elastic and the viscous shear modulus of all the independent data sets was calculated, giving rise to an indication of whether the behaviour of the sample was more elastic or more viscous, with > 1 being more elastic and < 1 being more viscous. In addition, the results were plotted in a loglog scale showing the mean and the standard error.
3) All the log-transformed independent data sets were checked for normality using the D'Agostino-Pearson K2 normality test. All the log-transformed independent data sets followed a normal distribution.
4) Finally, two 2-Way ANOVA tests were performed, one with all the independent data sets associated to the elastic shear modulus and the other one with all the independent data sets associated to the viscous shear modulus, with the two dependent variables being the frequency and the condition. Significance was considered for $p < 0.05$.

5.4. Results

This section presents the results obtained for the experiments described in the methods and it discusses its implications.

5.4.1. Internalisation of beads

Figure 14 presents one of the cells with beads imaged with confocal microscopy. Figure 14A shows a brightfield image of the cell and it focuses on a bead in particular, which it is clearly inside the cell in the XY plane, but it is not clear whether it is inside the cell in the Z direction, that is, it could be beneath the cell or on top of it. Figure 14B shows the same cell with the addition of a staining for the nucleus and a staining for the actin cytoskeleton, which covers all the cytoplasm of the cell, and it further proves that the bead is within the cytoplasm, at least in the XY plane. It is in Figure 14C, which represents a projection of the bead in the YZ plane, that it is finally shown that the bead is placed in the same Z position as the rest of the cytoplasm, suggesting that the bead is inside the cell.

In addition, the intensity profile along the Z direction of both the bead and the actin signal at the location of the bead was measured. Figure 14D shows a typical intensity profile for these two signals. The bead and the actin signal have its maximum intensity in the same position, showing that they are placed in the same Z position. Moreover, the actin signal is wider than the bead signal, showing that the bead is contained by the actin cytoskeleton. Taken together, these results demonstrate that the beads were embedded in the cytoplasm of the cells.

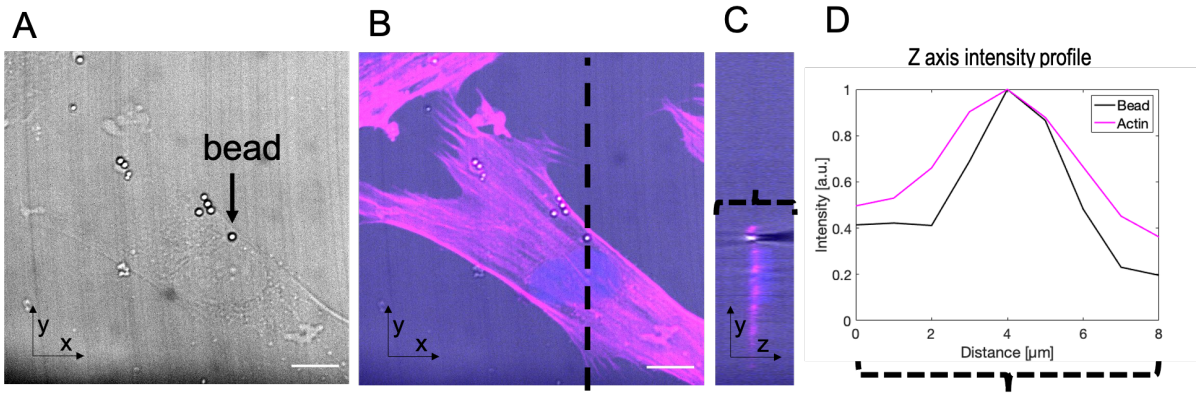

Figure 14. (A) Brightfield image of a cell with a bead. (B) The same cell stained with fluorescently labelled phalloidin (magenta) and Hoechst (blue). (C) YZ projection of the same cell along the dashed line. (D) Z intensity profile of the brightfield signal (bead) and the phalloidin signal (actin). The curly brackets indicate where the Z intensity profile was measured. Scale bars = 10 µm.

5.4.2. Acquisition of measurements optimisation

Figure 15 presents the complex shear modulus of the cytoplasm of TIFF fibroblast cells as a function of frequency measured with optical tweezers. One of the first things that can be observed is that, for the frequency range investigated, the elastic shear modulus dominates over the viscous shear modulus, indicating that the cytoplasm behaves more as a solid-like material than a fluid-like material. Furthermore, the complex shear modulus increases linearly in a loglog scale as a function of frequency. These findings

are consistent with previous reports in the literature investigating the viscoelastic properties of other cell types (12,25,27). Only in some specific cases, such as cells undergoing mitosis, in which the viscous component is known to dominate for frequencies larger than 2 Hertz, the behaviour is different. In addition, comparing these results with previously published findings, it has been observed that the stiffness of the cytoplasm of TIFF fibroblast cells is increased by approximately 2-fold in comparison with the stiffness of the cytoplasm of other cell types, such as A7 cells, measured with the same technique (12,25).

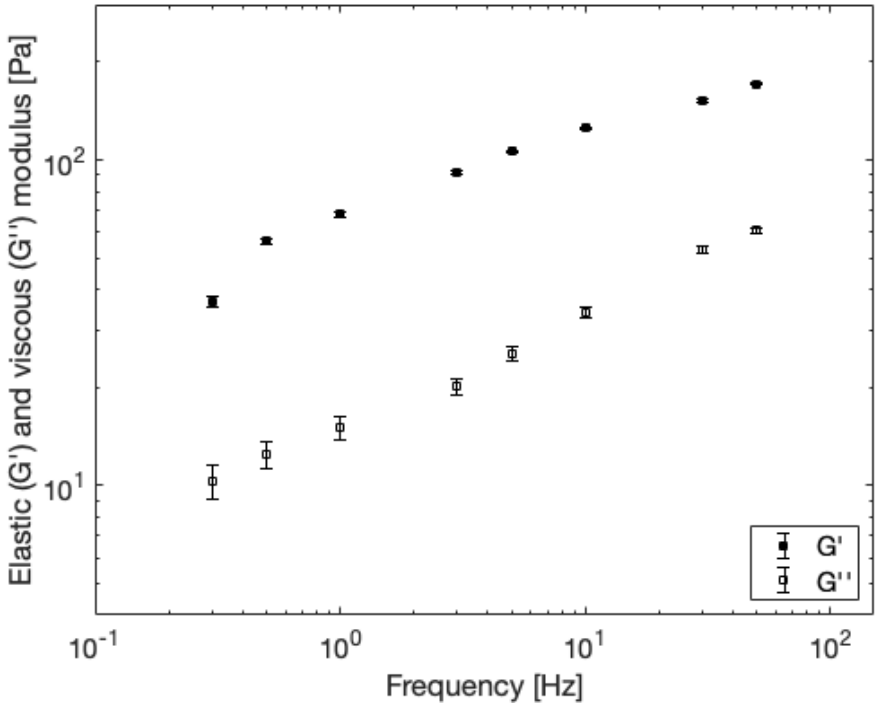

Figure 15. Complex shear modulus of the cytoplasm of TIFF fibroblast cells at different frequencies. Error bars represent the standard error (n = 24 cells from 2 independent experiments).

It is known that the viscoelastic properties of the cellular cytoplasm are dominated by the contribution of the actin cytoskeleton (12). Therefore, to verify that the system was directly measuring the complex shear modulus inside the cell, cells were treated with cytochalasin D, a drug that disrupts the actin cytoskeleton (33). Firstly, it was verified that cytochalasin D alters the actin organisation by fixing and staining cells treated with this drug. Figure 16A shows the organisation of the actin network in untreated TIFF fibroblast cells, whereas Figure 16B shows cytochalasin D treated TIFF fibroblast cells. These images clearly demonstrate that cytochalasin D triggers large-scale changes to the actin network and, therefore, it is a good control to verify that the microrheology experiments are directly probing the mechanical properties of the cytoplasm.

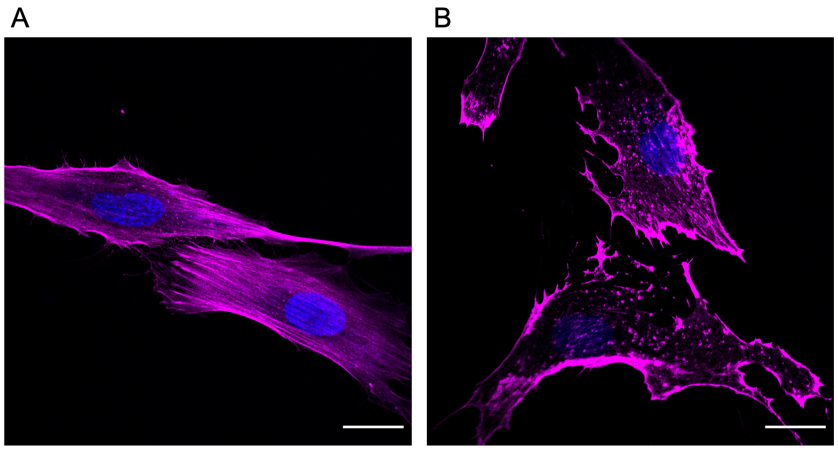

Figure 16. (A) Untreated TIFF fibroblast cells. (B) TIFF fibroblast cells treated with cytochalasin D for 30 minutes. Cells were fixed and stained with fluorescently labelled phalloidin (magenta) and Hoechst (blue). Scale bars = 20 µm. Images taken from Amy Beedle's project on fibrillar adhesions.

Performing microrheological measurements on cytochalasin D treated cells revealed that these cells exhibited a softer cytoplasm, with a decrease of both the elastic and the viscous component of by approximately 2-fold, as it is presented in Figure 17A. These results are consistent with previous reports in the literature for other cytoskeleton drugs (12). Moreover, the result of the 2-Way ANOVA tests indicates that the differences were statistically significant with $p < 0.001$ for both the elastic and the viscous component. Then, it was evaluated whether the relationship between the elastic and the viscous components was maintained between the control cells and the cells treated with cytochalasin D. This ratio provides information on which component, the elastic or the viscous, is more dominant in the cytoplasm. Plotting the ratio between the elastic and the viscous shear modulus for all frequencies revealed that in both cases it was greater than 1, as it is shown in Figure 17B, and, therefore, in both cases the behaviour was mainly elastic. Despite not altering this ratio, cytochalasin D significantly altered both the elastic and the viscous components and, therefore, these results demonstrate that the system was optimised to perform active microrheology experiments inside the cytoplasm of living cells. Furthermore, they reveal that the organisation of the actin cytoskeleton is a big determinant of the mechanical properties of the cytoplasm measured with this technique.

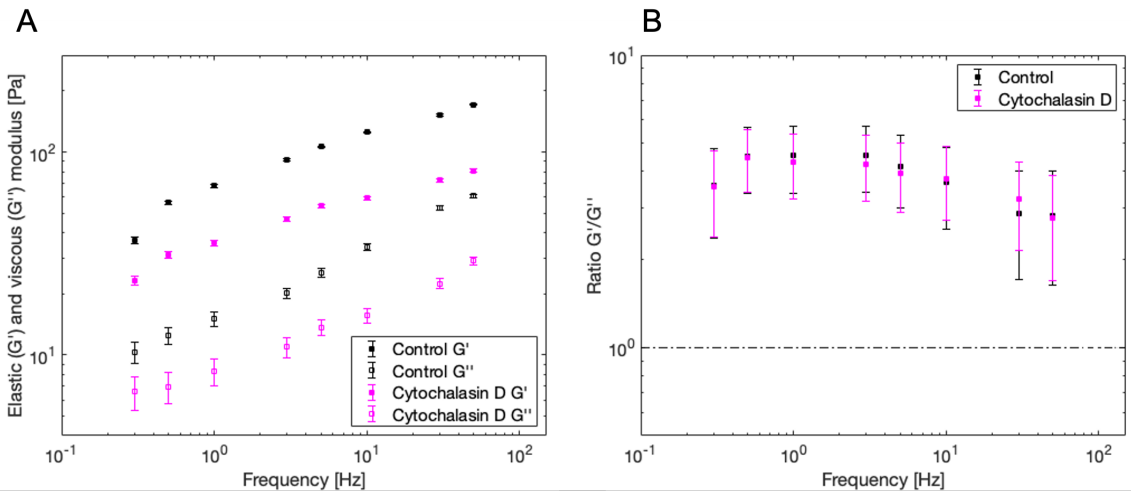

Figure 17. (A) Complex shear modulus of untreated and cytochalasin D treated cells. Cytochalasin D treatment reduces the cytoplasmic stiffness, the differences between the control and the condition are significant for both the elastic and the viscous

modulus ($p < 0.001$). Error bars represent the standard error (n = 24 cells from 2 independent experiments for control cells, n = 25 cells from 2 independent experiments for cytochalasin D treated cells). (B) The ratio between the elastic and the viscous shear modulus indicates that the elastic component dominates in both conditions since it is > 1.

5.4.3. Fibrillar adhesions experiment

Current work in the research group being conducted by Amy Beedle shows that cells that cannot form fibrillar adhesions have a different organisation of the actin network underneath the nucleus. Figure 18 shows the apical and the basal organisation of actin in control cells and in cells treated with glutaraldehyde, which prevents the formation of fibrillar adhesions. Whereas the apical actin organisation is maintained in both conditions, cells that form fibrillar adhesions present additional actin fibres in the basal side. The current hypothesis is that this slight alteration in the actin organisation could change how deformations applied to the cell membrane are efficiently transmitted to the nucleus, as it is suggested by the experiment with the cell stretching device. Therefore, the purpose of the experiment with fibrillar adhesions was to determine whether there are differences in the mechanical properties of the cytoplasm between cells with fibrillar adhesions and cells without fibrillar adhesions that can explain why the nucleus responds differently to mechanical stimuli applied at the level of the plasma membrane. With this purpose, the mechanical properties of the cytoplasm were measured in both conditions and with two different types of treatments that prevented the formation of fibrillar adhesions, a treatment with glutaraldehyde and a treatment with PFA.

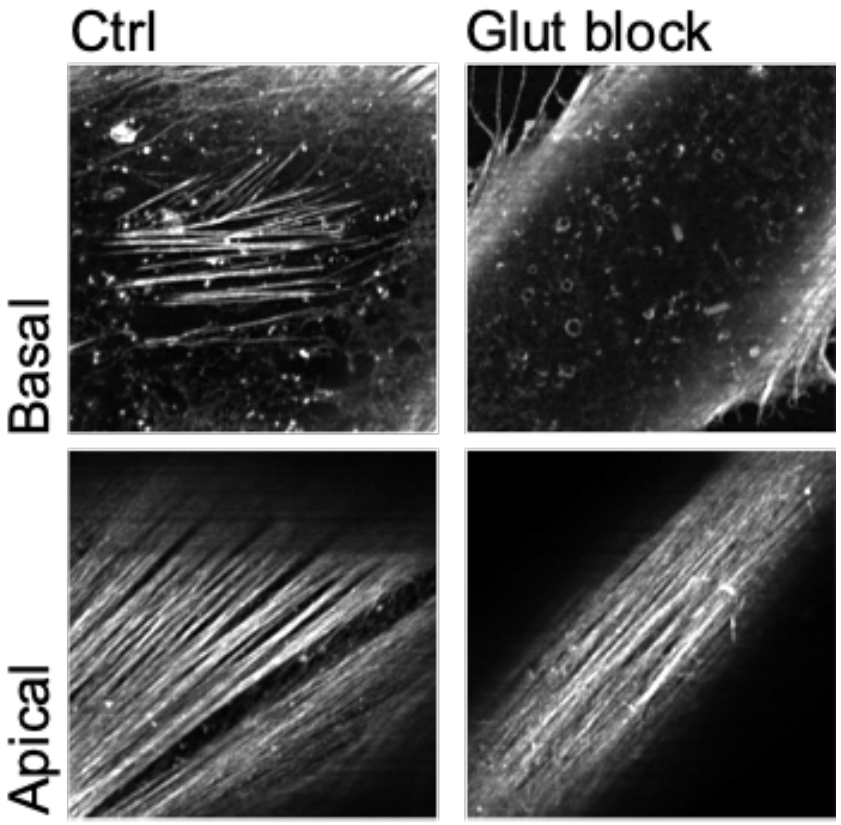

Figure 18. Apical and basal organisation of actin in control cells and in cells treated with glutaraldehyde, which prevents the formation of fibrillar adhesions. Whereas the apical organisation of actin is maintained, control cells present additional actin fibres in the basal side. Images taken from Amy Beedle's project on fibrillar adhesions.

The results regarding the glutaraldehyde treatment are presented in Figure 19A, whereas the results regarding the PFA treatment are presented in Figure 19B. In both cases, cells that were able to form fibrillar adhesions exhibited a softer cytoplasm. The 2-Way ANOVA tests indicated that for both treatments and for both the elastic and the viscous shear modulus the differences were statistically significant with $p < 0.001$. Regarding the ratio between the elastic and the viscous component, it did not change in any case (data not shown). However, it is worth mentioning that the experiment with the PFA treatment presents few data and, hence, for this treatment, the results are still preliminary.

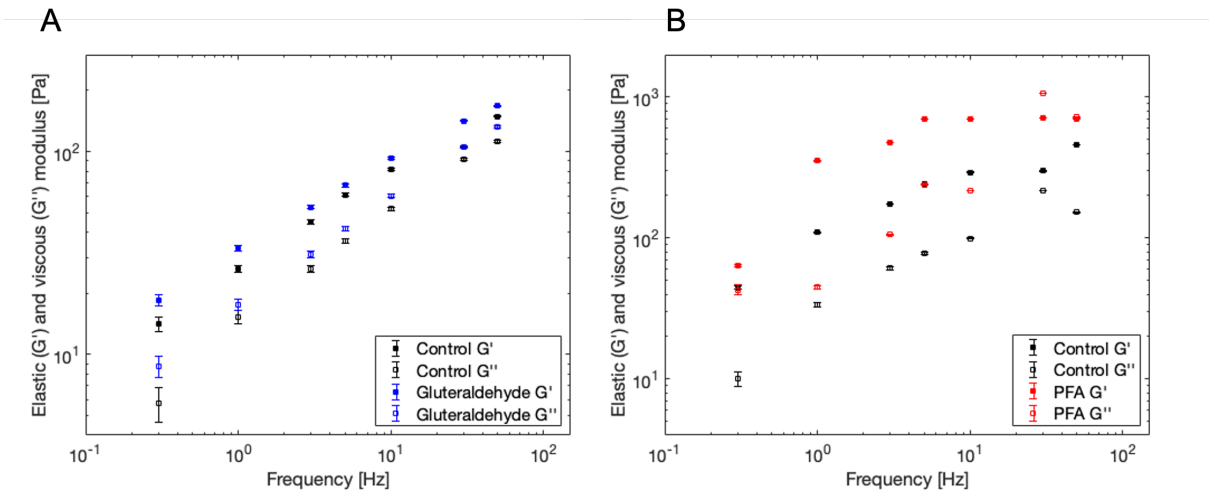

Figure 19. (A) Complex shear modulus of untreated and gluteraldehyde treated cells. Gluteraldehyde treatment increases the cytoplasmic stiffness, the differences between the control and the condition are significant for both the elastic and the viscous modulus ($p < 0.001$). Error bars represent the standard error ($n = 44$ cells from 2 independent experiments for control cells, $n = 36$ cells from 2 independent experiments for gluteraldehyde treated cells). (B) Complex shear modulus of untreated and PFA treated cells. PFA treatment also increases the cytoplasmic stiffness, the differences between the control and the condition are significant for both the elastic and the viscous modulus ($p < 0.001$). Error bars represent the standard error ($n = 8$ cells from 1 experiment for control cells, $n = 4$ cells from 1 experiment for PFA treated cells).

After having observed that the cytoplasm of cells with fibrillar adhesions is softer, the fact that the presence of fibrillar adhesions stops the deformations applied to the cell membrane from efficiently reaching the nucleus could be explained by the model presented in Figure 20. This model considers the cell as three springs in series, including the stiffness of the cytoplasm, the stiffness of the nucleus and, again, the stiffness of the cytoplasm. When fibrillar adhesions are present, the stiffness of the cytoplasm is lower and, when submitting cells to a certain strain, the cytoplasm absorbs most of this deformation. In contrast, when fibrillar adhesions are not present, the stiffness of the cytoplasm is higher and, when cells are submitted to the same strain, the cytoplasm deforms less, and the nucleus deforms more to compensate it. Furthermore, if the stiffness of the cytoplasm is lower, the effective stiffness of the whole cell is also lower, meaning that, when cells are submitted to the same strain, less force is transmitted throughout the cell.

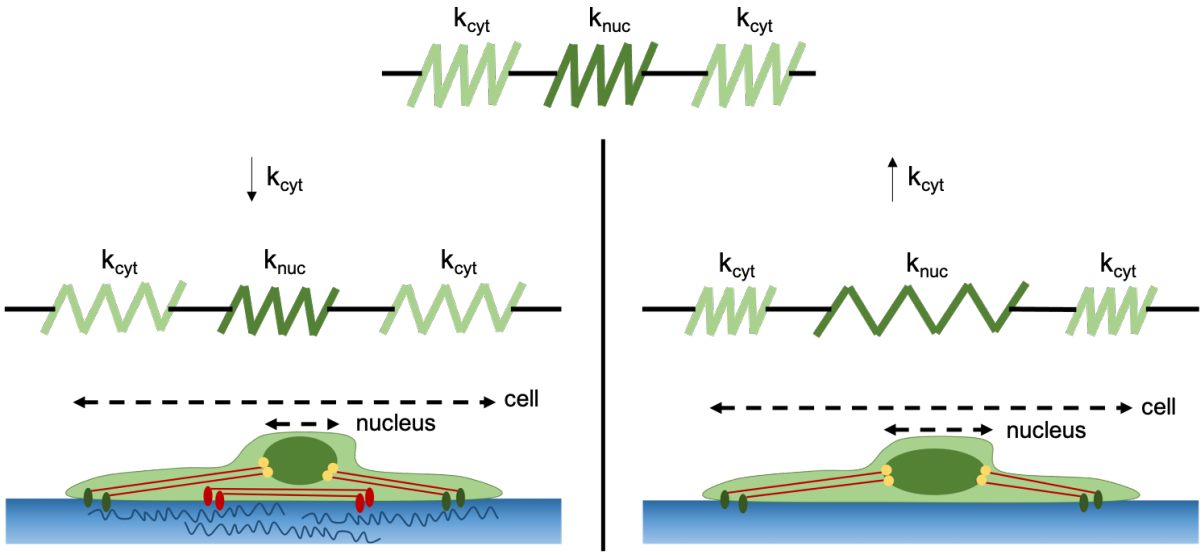

Figure 20. Model of the cell as three springs in series representing the cytoplasm and the nucleus. With fibrillar adhesions (left), the stiffness of the cytoplasm is lower, the cytoplasm deforms more upon stretching and the nucleus deforms less. Without fibrillar adhesions (right), the stiffness of the cytoplasm is higher, the cytoplasm deforms less upon stretching and the nucleus deforms more to compensate it.

In conclusion, fibrillar adhesions affect how mechanical stimuli applied to the cell membrane reach the nucleus and one of the mechanisms by which they do it may be by tunning the mechanical properties of the cytoplasm through the presence of additional actin fibres underneath the nucleus. This is further supported by the fact that it has also been proved that the actin cytoskeleton is a big determinant of the mechanical properties of the cytoplasm. However, although this reasoning can explain the results observed in the experiments, there are several aspects that have not been taken into account, the most important one being the stiffness of the nucleus itself. The stiffness of the nucleus could be affected by the presence of fibrillar adhesions, and this would also affect the forces and the deformations to which it is submitted. Therefore, in future studies, whether the stiffness of the nucleus is altered by fibrillar adhesions, which is still unknown, should be addressed.

6. EXECUTION SCHEDULE

This section describes the tasks in which the work was organised in order the accomplish the objectives. It also includes the time devoted to each task.

6.1. Task definition

The work was divided into four phases: the initiation phase, the concept generation phase, the execution phase and the closing phase. Throughout all its development, it was constantly supervised by the research director, Amy Beedle. Furthermore, before the closing phase, a meeting with the tutor and group leader of the Cellular and Molecular Mechanobiology Group, Pere Roca-Cusachs, was scheduled. The tasks corresponding to each phase are described hereunder.

INITIATION PHASE

The initiation phase took place from July 2020 to September 2020. It consisted in familiarising with the techniques and the current research in the Cellular and Molecular Mechanobiology Group.

1. **Background research**: it included reading literature about the current trends in mechanobiology and, specifically, on the mechanical role of fibrillar adhesions and the role of the nucleus as a mechanosensor. It also included reading literature about the physical principles of optical tweezers and their applications in the biological field, as well as literature about other biophysical tools to study cell mechanics.
2. **Laboratory training**: first, it included a training session about good laboratory practices. Then, it included trainings to learn cellular culture techniques and optical microscopy techniques. The cellular culture techniques learnt were thawing cells, passaging cells, transfecting cells, fixing cells and staining cells. Some of them were not used in the work presented. The microscopy skills learnt included trainings for two epifluorescence microscopes, one spinning disk confocal microscope and a Zeiss Airyscan microscope. Again, some of them were not used in the work presented. In addition, a first training for optical tweezers with the Impetux Optics team was performed. Finally, it included familiarising with the Fiji software and their applications for the analysis of microscopy images.

CONCEPT GENERATION PHASE

The concept generation phase took place during February 2021 and included defining the objectives of the work and selecting the ideas that were finally implemented. The technical and economic feasibility of the work were also explored.

3. **Definition of the objectives**: the main objective of the project and the specific objectives of the project were defined. They arose from the finding that fibrillar adhesions alter the mechanical coupling between the cell and the nucleus and the hypothesis that the mechanism by which they may do it is by tuning the mechanical properties of the cytoplasm.

4. **Selection of the solutions**: it included reading literature about optical tweezers as a microrheology technique to measure the mechanical properties of the cytoplasm and, from this information, propose and select the most suitable solutions to accomplish the objectives of the work. The most suitable method to internalise beads in the cytoplasm of the cells was defined to be phagocytosis of 1-micron beads. In addition, fixing, staining and imaging with confocal microscopy was selected to check that the beads were embedded in the cytoplasm of the cells. Moreover, cytoskeleton drugs and, specifically, cytochalasin D were selected as an optimal control to prove the correct functioning of the system.

EXECUTION PHASE

The execution phase took place during March, April and May 2021 and included implementing the selected solutions. In addition, once the system was optimised to perform active microrheology experiments inside the cytoplasm of living cells, the experiment with fibrillar adhesions was performed.

5. **Development of the bead's internalisation protocol**: several variations of the protocol to internalise beads inside the cytoplasm of the cells were tested, including different steps and different concentrations of beads. Simultaneously, samples were fixed, stained and imaged with confocal microscopy to determine whether the beads were inside the cytoplasm of the cells.
6. **Optimisation of the acquisition of measurements**: it included several training sessions with the Impetux Optics team in order to learn, specifically, how to perform active microrheology experiments inside the cytoplasm of living cells. From these training sessions, the optimal skills and parameters to manipulate the system were learnt. Then, microrheological measurements of the cytoplasm of untreated cells and cytochalasin D treated cells were performed.
7. **Experiment with fibrillar adhesions**: it included adapting the optimised protocol to internalise beads inside the cytoplasm of living cells in order to be able to study two cell conditions: with and without fibrillar adhesions. Two types of treatments to prevent the formation of fibrillar adhesions were tested: a glutaraldehyde treatment and a PFA treatment. In addition, it included the acquisition of the microrheological measurements of the cytoplasm for the two conditions.
8. **Data analysis**: a MATLAB program was implemented in order to read, plot and statistically test the measurements for the different cell conditions. A 2-Way ANOVA test was selected as the most suitable statistical test for the type of data obtained.

CLOSING PHASE

The closing phase took place during June 2021 and included the elaboration of a report and an oral presentation of the work. It also included a rehearsal of the oral presentation with the members of the Cellular and Molecular Mechanobiology Group.

9. **Project redaction**: the present report was redacted and delivered to a jury.
10. **Project presentation**: a presentation was prepared and defended in front of a jury.

6.2. GANTT diagram

In this section, the project schedule in the form of a GANTT chart with the specific time devoted to each task is provided (Figure 21). Previously, the legend of the chart is provided in Table 2.

Table 2. GANTT legend of the project.

GANTT LEGEND	
TASK ID	**DESCRIPTION**
1	Background research
2	Laboratory training
3	Definition of the objectives
4	Selection of the solutions
5	Development of the bead's internalisation protocol
6	Optimisation of the acquisition of measurements
7	Experiment with fibrillar adhesions
8	Data analysis
9	Project redaction
10	Project presentation

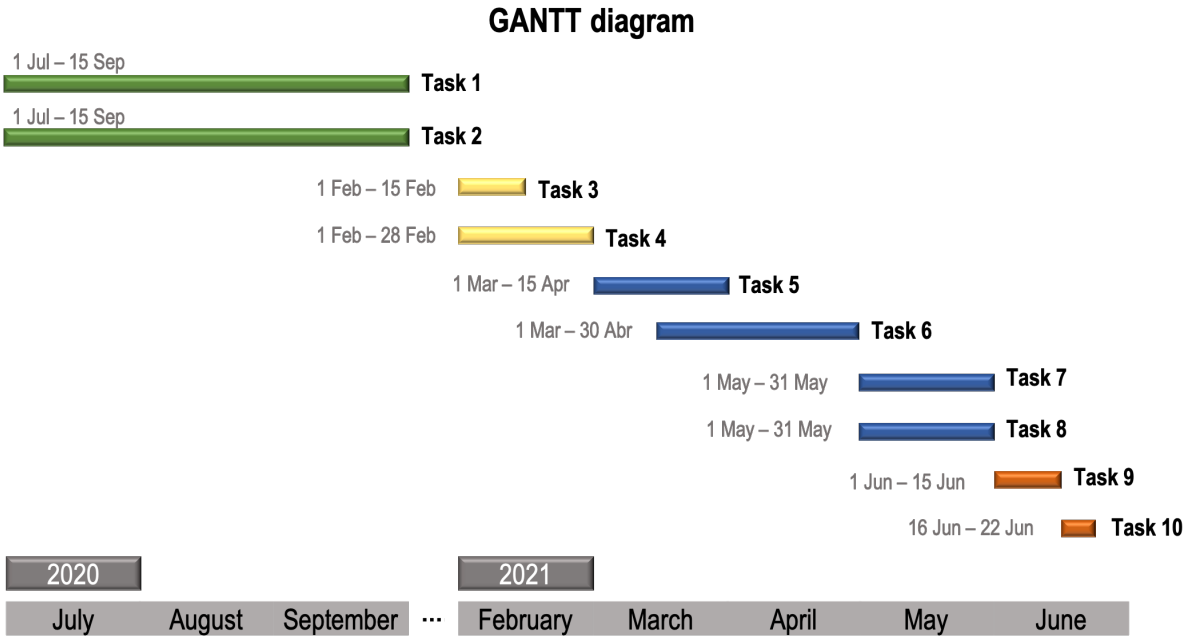

Figure 21. GANTT diagram of the project.

7. TECHNICAL AND ECONOMIC FEASIBILITY

This section discusses the technical and economic feasibility of the work presented.

7.1. Technical aspects

This work was entirely developed at the Cellular and Molecular Mechanobiology Group at the Institute for Bioengineering of Catalonia (IBEC). Therefore, the facilities, the equipment and the materials, including general laboratory material, reagents, microscopes and the optical tweezers setup, were provided by the research group. The detailed list of equipment and materials used was presented in *Detail engineering*.

In addition, the University of Barcelona provided a MATLAB 2021a (MathWorks) license and a Microsoft Office 2019 license, which were also necessary for the implementation of the MATLAB program and the project redaction and presentation, respectively. Taking everything into account, it can be stated that the project was technically feasible thanks to the contribution of the Cellular and Molecular Mechanobiology Group and the University of Barcelona.

7.2. Economical aspects

Table 3 presents the detailed costs of the project including the equipment, the materials, the software licences and the labour hours used. Regarding the equipment and the software licences, only the proportional cost of the elements is taken into account considering their initial cost and their lifetime. Regarding the materials, their cost corresponds to a pack or bottle, although in most cases it was not entirely used. General laboratory material appears simplified in a unique cost and it refers to pipettes, syringes, flasks, well-plates, gloves and a lab coat. All the materials used are commercially available except for the 3D printed metal chambers, which were designed by a previous member of the Cellular and Molecular Mechanobiology Group.

Finally, the labour cost is estimated regarding the hours that the research director and the undergraduate student dedicated to the project. However, given that this work is a bachelor's thesis, this cost was not an actual expense. Taking everything into account, it can be stated that the project was economically feasible, again, thanks to the contribution of the Cellular and Molecular Mechanobiology Group and the University of Barcelona. The Cellular and Molecular Mechanobiology Group provided funds for the equipment and the materials, and the University of Barcelona provided funds for the software licences.

Table 3. Detailed costs of the project.

PROJECT COSTS				
ITEM			PRICE (€)	%
Equipment	Optical tweezers platform SENSOCELL (Impetux Optics)	Annual price: 20,000€ Used time: 40h	390.00	3
	Inverted epifluorescence microscope (Eclipse Ti-e; Nikon)	Annual price: 30,000€ Used time: 40h	585.00	4

	Inverted microscope (Eclipse Ti-e; Nikon) with spinning disk confocal unit (CSU-WD; Yokogawa)	Annual price: 20,000€ Used time: 5h	70.00	0
	Objective (Plan Apo VC WI, 60x, NA=1.2; Nikon)	Annual price: 800€ Used time: 45h	16.00	0
	Cell culture inverted microscope (TS100; Nikon)	Annual price: 200€ Used time: 5h	0.70	0
	Biological hood (Telstar)	Annual price: 50€ Used time: 40h	1.00	0
	Cell incubator (Thermofischer Scientific)	Annual price: 50€ Used time: 8 months	38.00	0
	Cell culture centrifuge (Eppendorf Centrifuge 5702)	Annual price: 30€ Used time: 5h	0.10	0
	Benchtop centrifuge (Eppendorf MiniSpin)	Annual price: 20€ Used time: 1h	0.00	0
	Ultrasonic cleaner (VWR Ultrasonic Cleaners)	Annual price: 10€ Used time: 5h	0.20	0
	Fume hood equipped with nitrogen spray gun (Flores Valles)	Annual price: 50€ Used time: 5h	0.15	0
Materials	General laboratory material		50.00	0
	Dulbecco's modified eagle media DMEM (Thermofischer Scientific)	500 mL	49.81	0
	CO_2-independent media DMEM (Thermofischer Scientific)	500 mL	54.00	0
	Phosphate-buffered saline 1X (PBS) (Sigma Aldrich)	1L	90.50	1
	0.25% trypsin-EDTA (1X) (Gibco)	100 mL	16.84	0
	Neubauer chamber (Blaubrand)	Annual price: 10€ Used time: 5h	0.00	0
	Carboxylated 1 µm polystyrene beads (Micromod)	1 mL	93.70	1
	24 x 40 mm coverslips #1.5 (VWR) (Menzel-Gläser)	1x	64.45	0
	12 mm diameter coverslips (Paul Marienfield)	1x	14.28	0
	Fibronectin (Sigma Aldrich)	1 mg	157.00	1
	Bovine serum albumin (BSA) 10% in PBS (ThermoFischer Scientific)	50 mL	81.00	0
	Cytochalasin D (Sigma Aldrich)	1 mg	142.00	1
	Glutaraldehyde (Sigma Aldrich)	50 mL	145.00	1
	Paraformaldehyde (PFA) 4% in PBS (ThermoFischer Scientific)	500 G	41.90	0
	Triton X-100 (Sigma Aldrich)	6 · 10 mL	156.00	1

	Fish gelatin (Sigma Aldrich)	100 G	46.70	0
	Alexa Fluor 555 phalloidin (Thermo Fischer Scientific)	300 units	360.00	3
	Hoechst (Thermo Fischer Scientific)	5 mL	110.00	1
	Mowiol (ThermoFischer Scientific)	50 G	28.20	0
Office equipment	Computer equipped with MATLAB 2021a (MathWorks), Fiji (ImageJ), Mendeley, Microsoft Office and internet access.		800.00	6
Software licenses	MATLAB 2021a (MathWorks)	Annual license: 900€ Used time: 50h	25.00	0
	Microsoft Office 2019	Annual license: 100€ Used time: 50h	3.00	0
Labour	Research director	Price per hour: 25€ Used time: 250h	6,250.00	49
	Undergraduate student	Price per hour: 9€ Used time: 450h	4,050.00	29
TOTAL COST			**13,930.53**	**100**

7.3. SWOT analysis

This section presents a SWOT analysis (Figure 22), an analysis of the strengths, the weaknesses, the opportunities and the threats of the work presented.

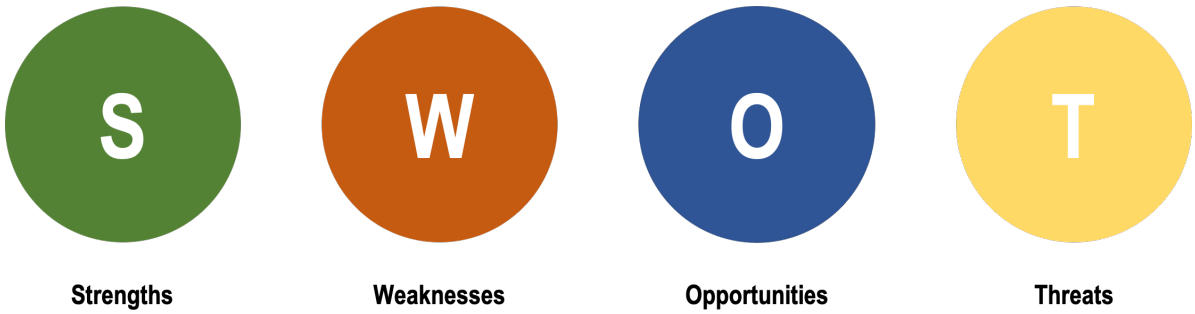

Figure 22. SWOT analysis.

Strengths: the work is based on optical tweezers, a non-invasive technique with high force, spatial and temporal resolution that is able to directly probe the mechanical properties of the cytoplasm. In addition, it was proved that the beads were inside the cytoplasm and that the cytoplasm was softer upon the addition of a cytoskeleton drug. Taken together, these results show that the optimised system is highly reliable. Moreover, the MATLAB program allows automatically displaying and analysing the data obtained with the optical tweezers setup. Regarding the experiment with fibrillar adhesions, the finding that cells with fibrillar adhesions exhibit a softer cytoplasm is compatible with previous work from Amy Beedle's project and suggests a mechanism by which fibrillar adhesions may alter the mechanical coupling between the cell and the nucleus.

Weaknesses: regarding the experiment with fibrillar adhesions, although the results for the glutaraldehyde treatment are reliable, the results for the PFA treatment are still preliminary due to scarce data. In addition, the model presented for the mechanical coupling between the cell and the nucleus assumes that the stiffness of the nucleus is not altered by the presence of fibrillar adhesions, and this is still not known.

Opportunities: optical tweezers as a microrheology technique to study the mechanical properties of the cytoplasm is an emerging field. There is already literature available, which allows comparing the results, but its capabilities remain to be fully explored, which makes this optimised protocol a very useful tool for future experiments. In addition, the current research in the field is mainly focused on how the mechanical properties of the cytoplasm affect the intracellular motion in different cell contexts, whereas this work proposes that the mechanical properties of the cytoplasm may also have an impact on the transmission of forces from the membrane of the cell to the nucleus, which is a slightly different approach. Moreover, it provides an insight into the mechanical role of fibrillar adhesions, which also remains to be unveiled.

Threats: currently, optical tweezers are also being used as a tool to measure the mechanical properties of the nucleus and, although the work performed allows internalising beads inside the cytoplasm of the cells, which would be the first step in order to mechanically probe the nucleus, it does not include an optimised protocol to do it.

8. LEGAL ASPECTS AND REGULATIONS

The work conducted was performed in a laboratory of research and involved the manipulation of hazardous materials, biological agents and human cells, specifically, TIFF fibroblast cells. Therefore, it required following the corresponding rules dictated by the European Commission and the Spanish Government concerning these aspects.

One the one hand, the Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 (36) regulates the protection of workers from risks related to exposure to biological agents at work. This is further regulated in Spain by the Royal Decree Law 664/1997 (37). On the other hand, the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 (38) sets the standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. This is further regulated in Spain by the Law 14/2007 (39) and the Royal Decree Law 1716/2011 (40).

It is also worth mentioning that, before starting the project, a training session about good laboratory practices organised by the Institute of Bioengineering of Catalonia was attended. These training included learning about the Spanish Law 31/1995 (41), which regulates the occupational risks prevention, and, specifically, its application in laboratories. It allowed being aware of the protocols to correctly handle chemicals and biological samples, to correctly manage laboratory waste and on how to react in case of an accident.

9. CONCLUSIONS AND FUTURE PERSPECTIVE

The aim of this work was to develop and optimise a protocol to study the mechanical properties of the cytoplasm using an optical tweezers setup as a microrheology technique and, in addition, test it for two different cell conditions, with and without fibrillar adhesions. Regarding the optimisation of the system, the protocol developed ensures the following:

1) Incubating cells with cell media and optical tweezers beads allows the internalisation of beads in the cytoplasm of cells by phagocytosis. Furthermore, the location of the beads was characterised by confocal microscopy and the results indicate that the beads were, in fact, in the cytoplasm.
2) Following the methods described in *Detail engineering* allows acquiring reliable microrheological measurements of the cytoplasm. The correct functioning of the system was proven by adding a control, a cytoskeleton drug that disrupted the actin cytoskeleton and induced a softening of the cytoplasm, consistent with previous studies in the literature.
3) Using the MATLAB program developed, the data acquired from the optical tweezers setup can be automatically read, displayed and statistically tested with a 2-Way ANOVA test, which allows determining whether there are statistically significant differences in the mechanical properties of the cytoplasm between different cell conditions.

Regarding the experiment with fibrillar adhesions, being able to measure the mechanical properties of the cytoplasm in the presence and in the absence of fibrillar adhesions has provided new insights into the mechanical role of fibrillar adhesions and, specifically, on how they may regulate the mechanical coupling between the cell and the nucleus. It has been observed that cells with fibrillar adhesions exhibit a softer cytoplasm, and it has been hypothesised that a softer cytoplasm is able to absorb larger deformations when a displacement is applied at the level of the cell membrane, leading to a shielding of the nucleus from these mechanical stimuli. This is compatible with what was observed in experiments performed with a cell stretching device in which it was shown that, with the presence of fibrillar adhesions, the strain on the nucleus is much lower than the strain of the cell, that is, the nucleus is more protected from these external mechanical perturbations.

Taking everything into account, it can be stated that the objectives of this work have been accomplished. However, it is also worth mentioning that the model proposed for the mechanical coupling between the cell and the nucleus has many assumptions, and one of the most important ones is that the stiffness of the nucleus is not altered by the presence of fibrillar adhesions. Interestingly, optical tweezers can also be used to directly probe the mechanical properties of the nucleus by deforming it with a bead. Therefore, and given that some of the beads embedded in the cytoplasm of the cells were already located close to the nucleus, it is clear that an interesting future perspective would be to study the mechanical properties of the nucleus with optical tweezers.

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11. ANNEXES

11.1. MATLAB program

The code of the MATLAB program described in *Detail engineering* is presented hereunder. The number of each step presented in *Detail engineering* corresponds to the number of each section in the code, which are displayed in green. Briefly, it reads the data, and it calculates the mean and the standard error of the complex shear modulus and the ratio between the elastic and the viscous shear modulus for all the log-transformed independent data sets. Then, the data is checked for normality using the D'Agostino-Pearson K2 normality test and, finally, two 2-Way ANOVA tests with the frequency and the condition being the two independent variables are performed. The first one corresponds to the elastic data sets and the second one corresponds to the viscous data sets. In addition, for a better comprehension, all the variable names including *control* refer to the control data sets, whereas all the variable names including *condition* refer to the condition data sets.

```
% Read data
data = table2array(readtable("control.txt","NumHeaderLines",0));
data_condition =
table2array(readtable("condition.txt","NumHeaderLines",0));
num_frequencies_1 = 8;
num_frequencies_2 = 8;

% 1. Plot complex shear modulus
frequency_vector_1 = data(1:num_frequencies_1,1);
frequency_vector_2 = data_condition(1:num_frequencies_2,1);
elastic = reshape(data(:,4),num_frequencies_1,[]);
viscous = reshape(data(:,5),num_frequencies_1,[]);
elastic_condition = reshape(data_condition(:,4),num_frequencies_2,[]);
viscous_condition = reshape(data_condition(:,5),num_frequencies_2,[]);

elastic_means = 10.^(mean(log10(elastic),2));
viscous_means = 10.^(mean(log10(viscous),2));
elastic_means_condition = 10.^(mean(log10(elastic_condition),2));
viscous_means_condition = 10.^(mean(log10(viscous_condition),2));

elastic_stes = 10.^(std(log10(elastic),[],2)/sqrt(length(elastic)));
viscous_stes = 10.^(std(log10(viscous),[],2)/sqrt(length(elastic)));
elastic_stes_condition =
10.^(std(log10(elastic_condition),[],2)/sqrt(length(elastic_condition)));
viscous_stes_condition =
10.^(std(log10(viscous_condition),[],2)/sqrt(length(elastic_condition)));

errorbar(frequency_vector_1,elastic_means,elastic_stes,'s',"MarkerFaceColor
",'k',"MarkerSize",5,"Color",'k')
hold on
errorbar(frequency_vector_1,viscous_means,elastic_stes,'s',"MarkerSize",5,"
Color",'k')
errorbar(frequency_vector_2,elastic_means_condition,elastic_stes_condition,
's',"MarkerFaceColor",'m',"MarkerSize",5,"Color",'m')
errorbar(frequency_vector_2,viscous_means_condition,viscous_stes_condition,
's',"MarkerSize",5,"Color",'m')
xlabel('Frequency [Hz]',"FontSize",15)
ylabel("Elastic (G') and viscous (G'') modulus [Pa]","FontSize",15)
```

```
legend(["Control G'","Control G''","Condition G'","Condition
G''"],"FontSize",15,"Location",'southeast')
set(gca,'YScale','log');
set(gca,'XScale','log');
xlim([0.1 150])
ylim([4 300])
ax=gca;
ax.XAxis.FontSize = 15;
ax.YAxis.FontSize = 15;
hold off

% 2. Plot ratio elastic and viscous shear modulus
ratio_means = 10.^(mean(log10(elastic./viscous),2));
ratio_stes = 10.^(std(log10(elastic./viscous),[],2)/sqrt(length(elastic)));
ratio_means_condition =
10.^(mean(log10(elastic_condition./viscous_condition),2));
ratio_stes_condition =
10.^(std(log10(elastic_condition./viscous_condition),[],2)/sqrt(length(elas
tic_condition)));

errorbar(frequency_vector_1,ratio_means,ratio_stes,'s',"MarkerFaceColor",'k
',"MarkerSize",5,"Color",'k')
hold on
errorbar(frequency_vector_2,ratio_means_condition,ratio_stes_condition,'s',
"MarkerFaceColor",'m',"MarkerSize",5,"Color",'m')
plot(0.1:150,ones(150),'-.k')
xlabel('Frequency [Hz]',"FontSize",15)
ylabel("Ratio G'/G''","FontSize",15)
legend(["Control","Condition"],"FontSize",15,"Location",'northeast')
set(gca,'YScale','log');
set(gca,'XScale','log');
xlim([0.1 150])
ylim([0.5 10])
ax=gca;
ax.XAxis.FontSize = 15;
ax.YAxis.FontSize = 15;
hold off

% 3. D'Agostino-Pearson K2 normality test
for frequency = 1:num_frequencies_1
    DagosPtest(log10(elastic(frequency,:)));
end
for frequency = 1:num_frequencies_2
    DagosPtest(log10(elastic_condition(frequency_test,:)));
end

% 4. 2-Way ANOVA test
num_cells = length(elastic(1,:));
num_cells_condition = length(elastic_condition(1,:));
condition_group = [repmat({'control'},[num_cells*length(frequency_vector_1)
1]);repmat({'condition'},[num_cells_condition*length(frequency_vector_2)
1])];
frequency_group = strings((num_cells)*num_frequencies_1,1);
frequency_group_condition =
strings((num_cells_condition)*num_frequencies_2,1);
for cell = 1:num_cells
    for frequency = 1:num_frequencies_1
        frequency_group((cell-1)*num_frequencies_1+frequency) =
repmat({sprintf('%d',frequency_vector_1(frequency))},[1 1]);
    end
end
```

```
for cell = 1:num_cells_condition
    for frequency = 1:num_frequencies_2
        frequency_group_condition((cell-1)*num_frequencies_2+frequency) =
repmat({sprintf('%d',frequency_vector_2(frequency))},[1 1]);
    end
end
frequency_group_all = [frequency_group;frequency_group_condition];
data_elastic_all = [log10(data(:,4));log10(data_condition(:,4))];
data_viscous_all = [log10(data(:,5));log10(data_condition(:,5))];
anovan(data_elastic_all,{frequency_group_all
condition_group},'model',2,'varnames',{'Frequency','Condition'})
anovan(data_viscous_all,{frequency_group_all
condition_group},'model',2,'varnames',{'Frequency','Condition'})
```